AD NUMBER

AD322280

CLASSIFICATION CHANGES

TO: unclassified

FROM: secret

LIMITATION CHANGES

TO:

Approved for public release, distribution unlimited

FROM:

DoD Controlling Organization: Army Biological Labs, Fort Detrick, Frederick, MD.

AUTHORITY

Dugway Proving Ground ltr, 10 Dec 1979; Dugway Proving Ground ltr, 10 Dec 1979

AD 322 280

TECHNICAL REPORT
BWL 1

SCREENING STUDIES WITH VARIOLA VIRUS (u)

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NOX

OCTOBER 1958

U.S. ARMY CHEMICAL CORPS
BIOLOGICAL WARFARE LABORATORIES
FORT DETRICK

U. S. ARMY CHEMICAL CORPS RESEARCH AND DEVELOPMENT COMMAND
U. S. ARMY BIOLOGICAL WARFARE LABORATORIES
Fort Detrick, Frederick, Maryland

BWL Technical Report 1

SCREENING STUDIES WITH VARIOLA VIRUS (U)

Nicholas Hahon

Special Operations Division
DEPUTY COMMANDER FOR SCIENTIFIC ACTIVITIES

Project 4-04-14-004

October 1958

Fort Detrick Control
Number 57-FDS-1620

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By: James A. Kime
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Date: 28 October 1958

(U) FOREWORD

(U) The work described in Technical Report 1 was authorized under Chemical Corps Project 4-04-14-004, "Special BW Operations." It was started in January 1954.

(U) The author is indebted to Milton Ratner, Robert Louie and Edmund Kozikowski for technical assistance. Appreciation is expressed to Dr. Harold L. Baier for drying services, to Mr. Peter Demchak for aerosol testing, and to Dr. Benjamin J. Wilson for advice and suggestions.

(U) ABSTRACT

(U) Technical Report 1 describes an investigation initiated to screen variola virus. The experimental data are concerned with methods of assaying the virus quantitatively, determining optimal conditions for culture growth, and the effect of various physical and chemical reagents on its viability. Viability after lyophilization and the storage stabilities of both liquid and dried organisms were noted. Monkeys, as a test host, were infected by the parenteral, oral, intranasal, and aerosol routes. The disease picture in these animals was similar to that of human infections. The findings of viral cytopathogenicity and propagation of the organisms in different tissue cultures are given.

(S) DIGEST

(S) Technical Report 1 is concerned with the screening of variola virus as a potential BW agent. Of the seven variola virus strains obtained from cases of human infection, six were shown to be virulent for monkeys. The Yamada strain (one of the six) was selected as a model for investigation because of a high mortality history. The chorioallantoic membrane (CAM) inoculation of 11- to 13-day-old embryonated eggs proved best for evaluating the potency of virus preparations by the pock-counting method. Highest virus yields were obtained after inoculating the CAM of 9- to 13-day-old embryonated eggs with 50,000 infectious units in a volume of 0.05 milliliter and incubating at 35°C for 48 hours. The use of cortisone acetate and hyaluronidase did not increase virus yields, nor did treatment with ultrasonic vibration.

(C) Both liquid and dried agent preparations withstood repeated freezing and thawing without loss of viability. The virus, completely inactivated by one per cent Lysol and by one per cent formalin under test conditions, was only partially inactivated by 200 parts per million of Roccal, ethyl alcohol or Carboxide gas. A pH of 7.3 was optimal for maintaining stability of preparations. Lyophilizing and grinding in the Tanner Spin Mill did not adversely affect viability.

(U) Preliminary studies with aerosols showed that recovery was increased from approximately 14 to 62 per cent by using a suitable impinger fluid in the first sampling period.

(S) Monkeys, the animal of choice for determining virus infectivity, were infected by the parenteral, intranasal, oral, and aerosol routes. The disease pattern was similar to that of human infections. Febrile response, presence of pustules, isolation of virus, and immunological response were the criteria of infection. Both the hemagglutination-inhibition and serum neutralization tests proved acceptable for specific antibody detection. Preliminary data showed that the Macaca irus monkey was more susceptible than the Macaca rhesus by the aerosol route.

(C) The storage stabilities of the dried agent held at temperatures of -60°C and -25°C showed no significant loss of titer after 64 weeks. The viability loss was 0.9 log at 4°C storage and 4.0 log at 25°C after 64 weeks. A liquid CAM preparation of the agent maintained titer during storage at -60°C for 64 weeks. In contrast to the stable titers of dried agent, the liquid agent showed a 0.6 log loss at -25°C and an 8.0 log loss at 4°C after 64 weeks' storage. Virus in the liquid form stored at 25°C could not be detected after 32 weeks, showing an 8.0 log loss. The agent stored in the dried form had better stability than the liquid preparation when held at -25°C, 4°C and 25°C for 64 weeks.

(C) The agent was able to induce a cytopathogenic effect with multiplication in a variety of human and animal cell lines in tissue culture. Viral multiplication in tissue culture gave virus yields equivalent to those obtained from embryonated eggs and frequently were 1.0 log greater. A straight-line logarithmic correlation was shown to exist between different quantities of virus neutralized and serum dilutions. These findings offer the possibility of using tissue culture systems for viral production, assay and immunological studies.

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I. (S) INTRODUCTION

A. (S) SCOPE OF THE PROJECT

(S) The marked contagiousness and debilitating effect of variola (smallpox) among unvaccinated and occasionally vaccinated populations prompted its investigation. The project, whose purpose was to screen variola virus as a potential BW agent, had the following objectives:

1. Survey literature and collect data.

2. Acquire variola strains of known human virulence.

3. Quantitate methods of viral assay and immunological response; study the optimal conditions of virus culture and aerosol assay; determine the drying and storage stability potential of the agent; define the disease pattern in a susceptible host after aerosol exposure; and gather other pertinent information necessary for the evaluation of this agent.

4. Explore the use of tissue-culture techniques as a method of viral production and as a possible means of enhancing virulence.

B. (U) HISTORY

(U) Smallpox or variola is one of the oldest diseases known to mankind. Accounts were recorded in 1122 B. C. of an epidemic in China. The disease was known in ancient India and is believed to have occurred among prehistoric Negroes of Central Africa. Smallpox was introduced into the Western Hemisphere shortly after the voyages of Columbus; the African slave trade contributed to its continued prevalence in North America. In the eighteenth century, approximately sixty million lives were lost in Europe because of the disease. About 1789, Jenner's discovery that material from cowpox lesions could be inoculated artificially to induce resistance to smallpox sparked the use of vaccination.

(U) Variola minor, a less severe form of the disease, was prevalent in Britain after World War I until 1935 and, although now rare, sporadic cases still occur. Several importations into this country of variola major, the more severe form, occurred during and after World War II. In 1946, the disease was brought to the Puget Sound area by military personnel returning from overseas. The presence of variola major in Mexico and Central America requires constant vigilance to prevent importation. In 1947, smallpox was diagnosed in a New York City man who had dealt with hundreds of people before his illness was properly diagnosed. Eleven secondary cases resulted. Numerous cases and outbreaks are recorded continually throughout the world where the disease in endemic.

(U) The California State Department of Public Health recently surveyed the smallpox immunization levels of persons living in the State. 1/* The information, based on a sample of about 3,500 households, represents the entire state, not any particular area. Of sampled children under six, 40 per cent had never been vaccinated against smallpox. Better protection against smallpox was found in the three- to five-year-olds than in young adults (20 to 24 years old), where only about 35 per cent were effectively immunized. For most other adult age groups, only 10 to 15 per cent appeared to be effectively immunized. This varying low percentage in a progressive, health-conscious state may indicate a still lower immunization level in other states and in foreign countries.

C. (U) ETIOLOGY

(U) Variola virus is a member of the exanthema (eruption upon the skin) diseases which include measles, rubella and varicella. Fluid from variola lesions contains numerous elementary bodies (sometimes called Paschen bodies) which are small, spherical structures with a diameter of about 200 microns. This elementary body is assumed to represent the virus particle.

(U) Variola is highly infectious and is transmitted by contact either with the patient or his immediate surroundings. The virus, reportedly quite stable, retains its infectivity for a year in dust and fomites.

D. (U) CLINICAL PICTURE OF DISEASE

(U) The typical picture of smallpox: twelve days after exposure to variola virus, the patient develops chills, prostration, headache, severe backache, vomiting and fever of about 103°F. These prodromata continue for three to four days, during which time a fleeting rash of indefinite type may be detected. At the end of this period, eruption appears and temperature falls close to normal. The rash begins as a discrete papular eruption on the face, rapidly appears on the extremities and trunk, and may become confluent in many areas. The pustules begin to dry and generally are crusted over by the end of the second week; the scabs drop off at the end of the third week. Practically all of the lesions in one area simultaneously undergo transition from the papular to vesicular and thence to the pustular stage. With the onset of the pustular stage, fever generally returns and persists for several days at a level of 101° to 102°F. A mild leukopenia (decrease in the normal number of leukocytes in peripheral blood) often occurs during the pre-eruptive stage while a moderate leukocytosis (increase in normal leukocyte count) appears with pustulation.

(U) The case mortality rate of discrete variola is about 10 per cent, for the confluent form, about 50 per cent, and more than 80 per cent for the hemorrhagic type. There is universal susceptibility unless a person has been recently vaccinated or has experienced the disease.

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* See Literature Cited.

II. (S) MATERIALS AND METHODS OF STUDY

A. (S) VIRUS STRAINS

(S) The seven strains of variola virus obtained for study were identified as Yamada, Lee, Kim, Harper, Stillwell, Gassman, and Hartrige. All except the Hartrige strain were obtained through the courtesy of Dr. Joseph E. Smadel, Army Medical Service Graduate School, Washington, D.C. The Hartrige strain was obtained from Dr. Karl Habel, National Institutes of Health, Bethesda, Maryland. All the strains were either in the second or third egg passage. A history of each is presented in Table I. All strains were made to a 10^{-3} dilution and passed three times on the chorioallantoic membrane (CAM) of 11- to 12-day-old White Leghorn embryonated eggs. The first passage was considered a master pool, the second the stock pool and the third passage a working pool. Whenever a working pool was exhausted, a virus sample of the stock pool was passed in eggs to make another working pool. In this manner, all experimental studies with the virus were in the same CAM egg passage.

(S) Unless otherwise indicated, the data compiled in experimental studies were obtained from the Yamada strain. This strain was selected for its immediate availability and high mortality for man*. In addition, Yamada was as infectious for monkeys by the parenteral and intranasal routes of inoculation as were the other variola strains. The Yamada strain was used as a model for the disease until more virulent strains were obtained, or until those available could be properly evaluated for their disease-producing qualities.

B. (U) IDENTIFICATION OF VARIOLA VIRUS

(U) Of necessity, the Yamada strain of variola virus under study had to be distinct from any contaminating virus, such as vaccinia, which it closely resembles. Immunological tests were of limited value because both viruses possess certain similar antigenic components which give cross-reactions. To distinguish variola from vaccinia, certain animal passage characteristics and pock lesion morphology were used:

1. Serial passage in rabbit skin - Characteristically, passing variola from the skin of one rabbit to another is difficult. Variola, inoculated intradermally, provokes a cutaneous response within 48 hours. The red, firm nodule resulting from the reaction (Figure 1) is excised, homogenized, and inoculated intradermally into the skin of a second rabbit. It provokes no cutaneous response if the primary reaction was due to variola virus. An excised vaccinia lesion will continue to cause a cutaneous reaction on serial passage in the skin of a rabbit.

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* Personal communication to author from Lt. Col. A. S. Benenson, MC, USA, April 1955.

TABLE I. (S) HISTORY OF VARIOLA VIRUS STRAINS

Virus [a] Strain	No. of CAM Passages	Date of Last Passage	Physical State of Sample	Case History
Yamada	2	July 1946	Frozen Suspension	Japanese patient with moderately severe smallpox
Lee	2	11 Feb. 1947	Lyophilized	Isolated from vesicular fluid on the 7th and 8th day of disease from infants in Korea
Kim	2	11 Feb. 1947	Lyophilized	Isolated from vesicular fluid on the 7th and 8th day of disease from infants in Korea
Gassman	3	30 Sept. 1952	Frozen Suspension	Isolated by 406th Med. Gen. Lab. in Tokyo in 1951
Harper	3	3 Oct. 1952	Frozen Suspension	Isolated by 406th Med. Gen. Lab. in Tokyo, Fatal Case
Still-well	3	3 Oct. 1952	Frozen Suspension	Isolated by 406th Med. Gen. Lab. in Tokyo. Fatal Case
Hart-rige	Unknown	Unknown	Frozen Suspension	Unknown

a. Received at BW Laboratories latter part of 1953 and early 1954.

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1. Intradermal inoculation of variola virus of low titer. Small scab formed.
2. Variola virus of low titer scarified on the skin. No response.
3. Intradermal inoculation of high-titer variola virus. Large, red, raised, circumscribed nodule.
4. High-titer variola virus inoculated by scarification. No response.
5. Control - Suspension of normal chorioallantoic membrane, inoculated intradermally. No response.
6. Control - Suspension of normal chorioallantoic membrane, scarified. No response.

FIGURE 1. (U) CUTANEOUS RESPONSE IN RABBIT SKIN FOUR DAYS AFTER INOCULATION WITH VARIOLA VIRUS.

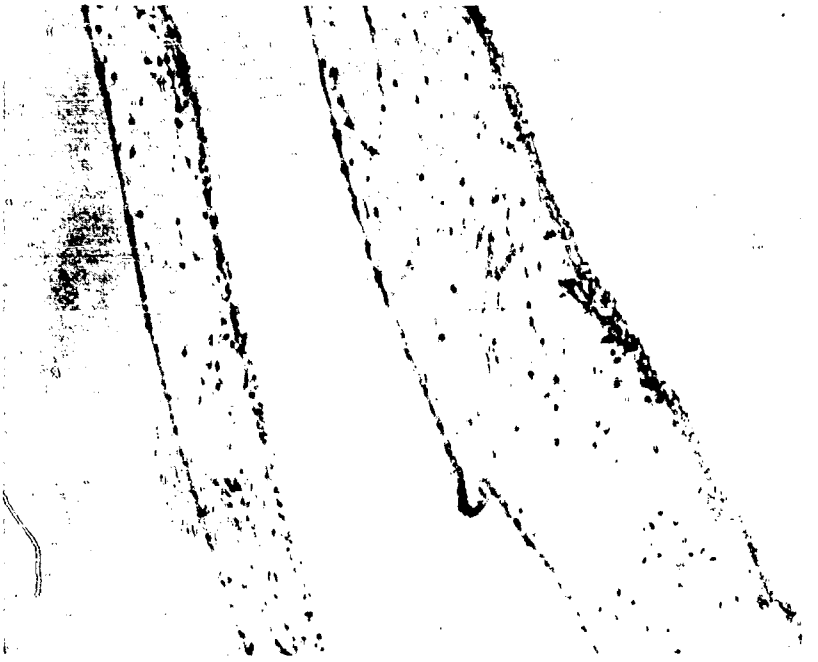


FIGURE 2. (U) NORMAL CAM OF 13-DAY-OLD EMBRYONATED EGG (H & E) X500. (NEG. NO. C-803)

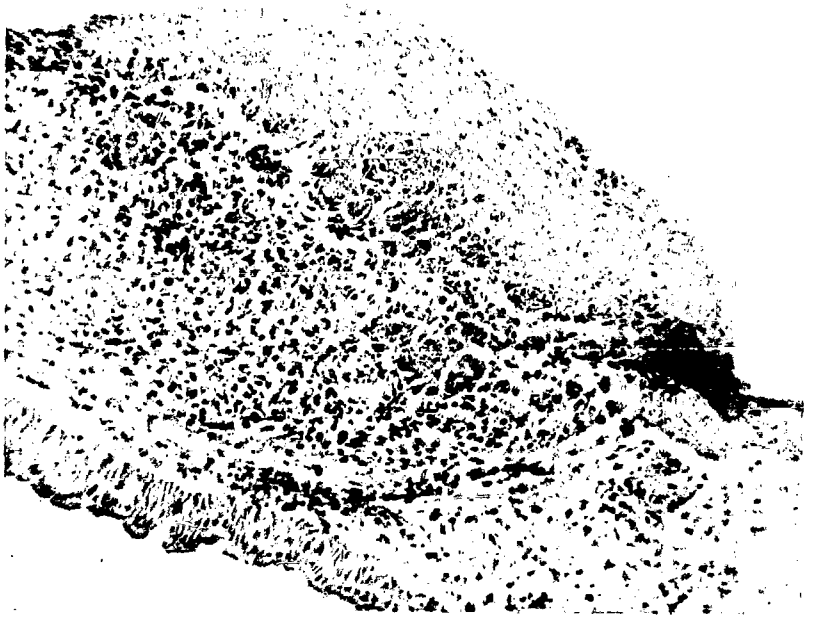


FIGURE 3. (U) VARIOLA-INFECTED CAM OF 13-DAY-OLD EMBRYONATED EGG (H & E) X500. (NEG. NO. C-802)

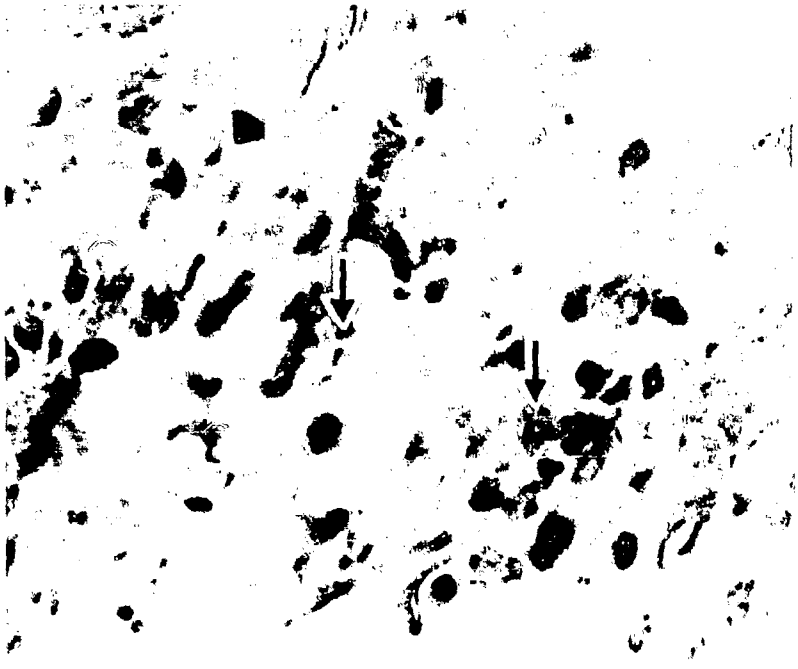


FIGURE 4. (U) VARIOLA-INFECTED CAM (MANN'S STAIN) X2250
ARROWS POINT TO INCLUSIONS.

2. Histological and morphological lesion differences - Variola lesions on the CAM of embryonated eggs are morphologically and histologically different from vaccinia lesions[2]. The lesions of vaccinia are much larger after three days' incubation than are those of variola. Isolated lesions of vaccinia may attain a diameter of three millimeters. They tend to be flatter and more necrotic, with a rough surface. Furthermore, hemorrhage into the membrane is more frequent, especially when the lesions are confluent. The lesions of variola are smaller and domed. Although with egg-adapted strains of vaccinia a heavy inoculum frequently causes fatal infection of the embryo, vaccinia virus from the human skin does not at first prove excessively virulent. The relative virulence of the viruses is of no great value in differentiation or diagnosis. Histologically, the vaccinial lesions are more extensive and more destructive than those produced by variola virus, and proliferation of the ectodermal cells is not as marked. Histological sections of normal and variola-infected CAMs stained with hematoxylin and eosin (H and E) are shown in Figures 2 and 3. The inclusion material in infected ectodermal cells has a similar appearance in the two types of infection, although, with variola, inclusions tend to be more granular. An inclusion of variola virus is shown in Figure 4.

3. Serial passage in mouse brain - An additional factor in differentiating the two viruses is their passage potential in mice. Mice injected intracerebrally with suspensions of variolous membranes die within a few days but with irregularity. Further passage of the virus causes no lethality of mice. Vaccinia, however, can readily be passed in mice generations.

(U) On the basis of the described characteristics, the Yamada strain was verified as variola.

C. (U) TECHNIQUE FOR PREPARING EGGS FOR CAM INOCULATION

(U) Modifications of conventional methods of CAM inoculation were introduced to avoid nonspecific lesions resulting from trauma[3-10]. By conventional inoculation, the lesions varied from large enough to obscure the field for counting to small enough to be mistaken for specific lesions. Their presence could constitute a source of variation in pock enumeration and thereby influence the accuracy of pock titrations[9,11,12,13].

(U) The modified method reduced nonspecific lesions from 70 to four per cent. Eggs were prepared for CAM inoculation by the usual procedure but with this additional feature: the artificially formed air space was rotated to a new area of the CAM. Nonspecific lesions which generally occurred in the area were the CAM was initially dropped were absent in the new CAM area.

(U) Steps in the modified method are as follows:

1. Eggs were candled and a circle ten millimeters in diameter was drawn on the side of each egg where the CAM was well developed. Areas over large blood vessels were avoided. The shells of the eggs were not disinfected.

2. A small hole was drilled through the shell over the center of the air space with a Vibro-Tool (Burgess Vibrocrafters Inc., Grayslake, Ill.) as shown in Figure 5.

3. The circle drawn on the egg was outlined with the Vibro-Tool (Figure 6). The circular cut was drilled just deep enough to loosen the flap of shell without penetrating the shell membrane.

4. The circular shell flap was detached from the shell membrane with a half-spear-point dissecting needle (Figure 7).

5. A drop of sterile saline was placed on the exposed shell membrane and a slit one to two millimeters long was made in the fibers of the membrane with a sterile dissecting needle.

6. After the saline had been in contact with membranes for one to two minutes, an artificial air space was formed when the CAM was dropped by gentle suction with a rubber bulb at the hole drilled in the air space (Figure 8). This was done in the light beam of an egg candler so that the size of the air space could be seen and controlled. The artificial air space was usually 20 to 25 millimeters in diameter.

7. The egg was rotated as soon as the artificial air space was formed. The newly formed air space was moved from the site of preparation to a well-developed area of the CAM. This rotation was made to the left or right depending on the location of the area of the CAM. The position of the artificial air space was then outlined.

8. A minute opening was drilled in the shell with the Vibro-Tool, over the area of the artificial air space (Figure 9) for introduction of inoculum.

9. An inoculum of 0.05 milliliter (ml) was introduced into the egg (Figure 10) from a one-ml Luer-Lok tuberculin syringe fitted with a 26- or 27-gauge, $\frac{1}{4}$-inch needle. The inoculated eggs were tilted slightly to distribute the inoculum evenly over the dropped area of the CAM. The eggs were then placed on their long axes in trays with the artificial air space uppermost and incubated undisturbed at 35°C for 72 hours. Leaving the openings in the shell unsealed in no way affected the viability of the egg or the formation of pocks.

FIGURE 5. (U) DRILLING AN OPENING TO THE AIR SPACE.

FIGURE 6. (U) DRILLING A CIRCULAR FLAP IN THE SHELL.

FIGURE 7. (U) REMOVAL OF THE CIRCULAR SHELL FLAP, EXPOSING THE SHELL MEMBRANE.

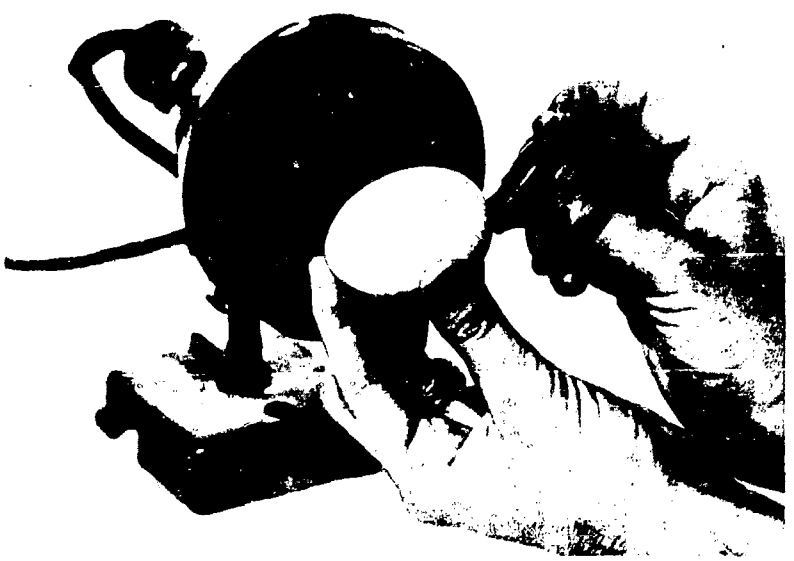


FIGURE 8. (U) FORMATION OF ARTIFICIAL AIR SPACE.

FIGURE 9. (U) DRILLING AN OPENING IN THE SHELL DIRECTLY ABOVE THE ROTATED AIR SPACE.

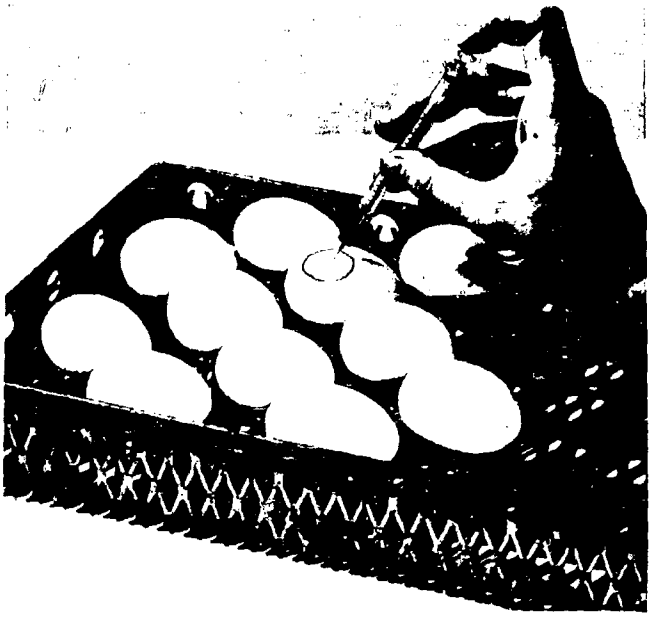


FIGURE 10. (U) INOCULATION OF THE CAM OVER THE ROTATED AIR SPACE.

(U) A few precautionary measures are important in this method. The slit in the shell membrane should be as small as possible, since the shell membrane is used as a natural seal when the eggs are rotated. In addition, in Step 6, the artificial air space should be formed not later than five minutes after the application of the saline. Experience shows that the exposed membranes dry rapidly. With prolonged delay the artificial air space is difficult to form and often irregular in shape.

D. (U) PROCEDURE FOR TITRATIONS

(U) Viruses were titrated as follows: Serial tenfold dilutions ranging from 10^{-1} to 10^{-6} were made in heart infusion broth (HIB) which contained 500 units of penicillin and 100 micrograms of streptomycin per milliliter. Generally, 0.05-ml volumes of 10^{-4}, 10^{-5}, and 10^{-6} virus dilutions were inoculated on the CAM of 11- to 13-day-old embryonated eggs. Preliminary testing of a standard virus preparation on embryonated eggs of different ages revealed that 11- to 13-day-old eggs were most sensitive for virus titration (Table II). Seven or eight eggs were inoculated per dilution and incubated at 35°C for 72 hours.

TABLE II. (U) INFLUENCE OF AGE OF EMBRYONATED EGGS ON VARIOLA VIRUS TITRATIONS

Age of Embryonated Egg, Days	Infectious Units per ml
6	26
7	50
8	1.8×10^6
9	1.2×10^7
10	1.3×10^7
11	6.4×10^7
12	6.6×10^7
13	6.6×10^7
14	2.1×10^7
15	2.4×10^7

E. (U) HARVESTING OF CAMS

(U) Inoculated CAMs were harvested by cutting the eggs in half along the long axis with curved scissors. The CAMs were detached from the egg halves containing the membranes with forceps, and washed twice in formal saline (ten per cent formalin in saline solution). The membranes were floated in Petri dishes containing formal saline and examined. When the membranes were harvested for virus pool production, aseptic procedures were used. The washing of membranes was omitted.

F. (U) EXAMINATION OF MEMBRANES

(U) Variola virus produces small foci; the chief features of the specific lesions are (a) an almost circular form, (b) a central, more opaque area of necrosis, and (c) a surrounding haze due to the inflammatory reaction in the mesodermal layer. As a good working rule, the lesion was considered specific when any two of these features were present.[7] A normal and a variola-infected membrane are shown in Figures 11 and 12.

(U) The pocks on infected membranes were counted with the aid of an illuminator. This instrument, devised to facilitate the examination of membranes, passes reflected light across a Petri dish containing infected membranes and illuminates the pocks by the darkfield principle.

(U) This type of illuminator offered several advantages. It diminished the handling of excised membranes, because the membranes needed only to be placed in a Petri dish with saline or formal saline and examined. The limited orientation of the membranes decreased tears and injury to the tissue. The examination could be done easily under sterile conditions if the infected membranes were to be used for virus pool production. The membranes in Petri dishes could be read either immediately or hours later without danger of drying. Pocks on the membranes were readily visible and easily distinguished from nonspecific lesions, which increased the accuracy of the pock count. The illuminator is shown in Figures 13 and 14.

G. (U) CALCULATION OF INFECTIOUS UNITS

(U) The number of infectious units (IU) per milliliter of suspension was determined by averaging the number of pocks per membrane, multiplying by 20 (for conversion to a milliliter basis) and by the dilution factor. Usually seven or eight membranes were counted per dilution. The membranes employed to determine the concentration of infectious units were those which had between zero and one hundred pocks.

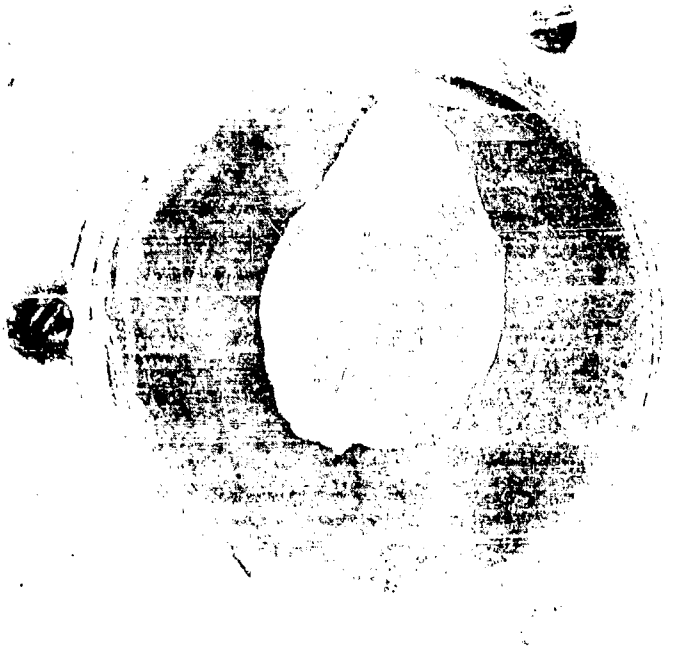


FIGURE 11. (U) NORMAL CAM AS VIEWED ON THE ILLUMINATOR.

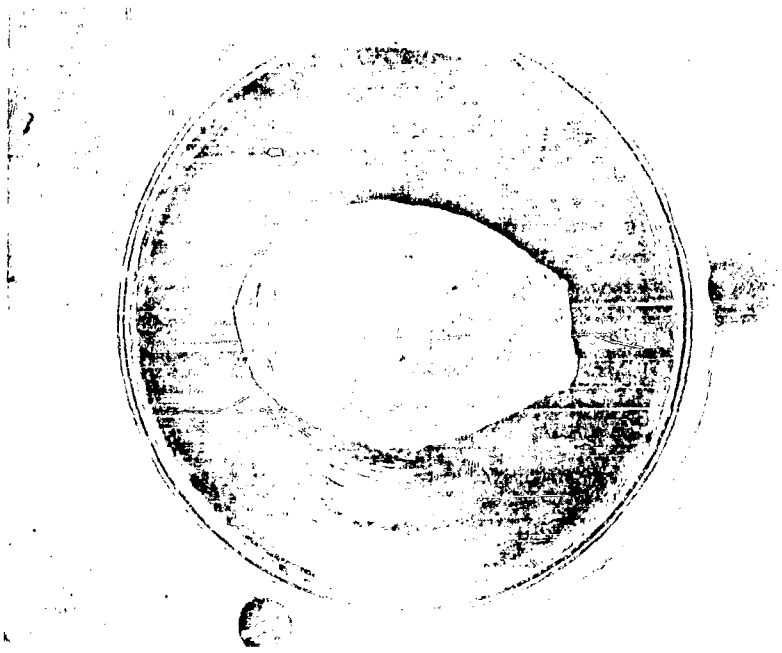


FIGURE 12. (U) VARIOLA-INFECTED CAM AS VIEWED ON THE ILLUMINATOR.

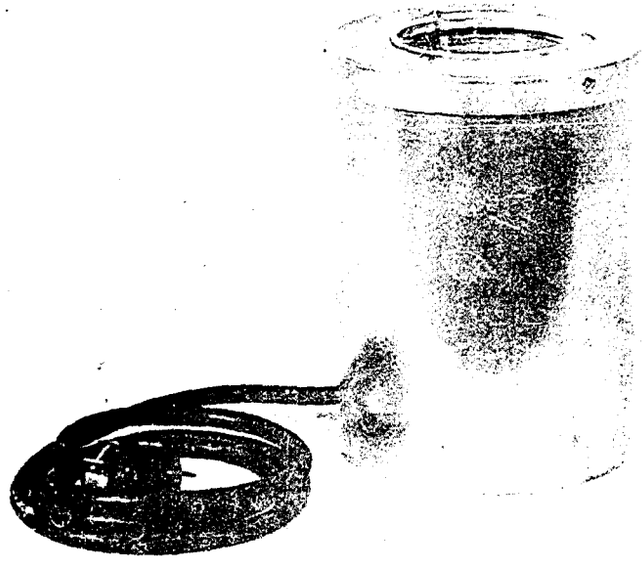


FIGURE 13. (U) ILLUMINATOR FOR CAM POCK ENUMERATION.

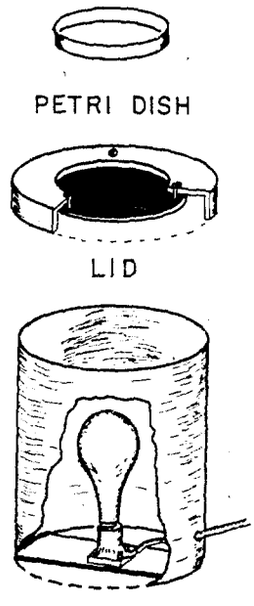


FIGURE 14. (U) DIAGRAM OF ILLUMINATOR.

H. (U) ACCURACY OF THE METHOD OF TITRATION

(U) This technique of CAM inoculation was tested for accuracy of viral titration by comparing it with the conventional method of dropping the CAM, which does not involve rotation of the artificial air space. Forty-two titrations, 21 with each technique, were made to test the difference between the two techniques. One virus pool, Yamada strain, was used throughout the test and the dilution, inoculation, incubation, and pock counting techniques previously described were used.

(U) Results obtained by the two methods and statistical calculations are shown in Table III. Different mean values were apparent for the techniques. The technique of rotation gave a higher mean. A test of significance was made to evaluate the difference between the two means. The t value was 4.580 for 40 degrees of freedom. The probability was less than one in one thousand that this value could occur by chance alone. Thus, the difference between the means was highly significant. The incidence of nonspecific lesions in the conventional or non-rotated treatment was 66 per cent higher than in the rotated treatment. It would appear that the rotated method of preparing eggs for CAM inoculation was more accurate for assaying viral suspensions.

I. (U) PREPARATION OF WORKING VIRUS POOLS

(U) Working virus pools were made by diluting an aliquot of the stock virus pool to a 10^{-2} suspension containing 50,000 infectious units per 0.05 milliliter. The 0.05 milliliter of inoculum was deposited on the CAM of 11- to 12-day-old embryonated eggs and incubated at 35°C for 48 hours. The infected membranes were then harvested and made into a 20 per cent suspension in HIB (pH 7.3), emulsified in a Waring Blendor for three minutes, and centrifuged for ten minutes at 2000 rpm in an angle-head centrifuge. The supernatant was stored in rubber-stoppered glass ampoules in an electrically operated freezer unit at -60°C.

J. (U) SEROLOGICAL TESTS

(U) The ability of the virus to elicit a specific antibody response was used as one means of detecting infection. The antibody response was studied by using serological tests to confirm infections of exposed or inoculated animals and to detect subclinical or asymptomatic infections. The serological tests successfully used for these purposes were the hemagglutination-inhibition (H-I) and the serum neutralization (SN) tests.

(U) The complement-fixation test was not attempted because of physical limitations in the laboratory. An intradermal neutralization test in rabbits was briefly investigated but did not prove satisfactory because of the inconsistency of the dermal response to a known infective virus.

TABLE III. (U) STATISTICAL EVALUATION OF TWO TECHNIQUES OF CAM INOCULATION USED FOR THE CALCULATION OF INFECTIOUS UNITS OF VARIOLA DERIVED FROM POCK COUNTS

TITRATION NO.	NON-ROTATED AIR SPACE	ROTATED AIR SPACE
	10^7 Infectious Units per ml	
1	6.0	5.4
2	3.6	7.6
3	8.6	10.0
4	9.4	11.0
5	5.0	4.0
6	4.8	9.2
7	3.6	11.0
8	5.0	8.6
9	5.8	6.0
10	5.0	6.2
11	2.4	8.0
12	2.2	6.2
13	3.4	5.2
14	1.4	5.2
15	4.0	9.8
16	6.0	6.8
17	4.2	8.4
18	2.8	7.2
19	6.0	5.4
20	4.8	11.2
21	5.2	7.4
Mean	4.7	7.6
Standard Deviation	$\pm$1.93	$\pm$2.14
Standard Error of Mean	$\pm$0.42	$\pm$0.46

1. (U) Hemagglutination-Inhibition Test

(U) Preliminary screening of the hemagglutination potential of the seven available virus strains showed all strains capable of agglutinating selected chicken erythrocytes. Since most of the experiments were made with the Yamada strain, it was used as the test antigen.

(U) Hemagglutination of erythrocytes by variola virus was dependent on the susceptibility of the chicken erythrocytes. An Australian group reports that approximately 50 per cent of tested fowls possess erythrocytes capable of being agglutinated by the virus [14]. SO Division found this value considerably lower. To locate fowls possessing susceptible cells, washed erythrocyte samples from fowls were tested and the agglutination pattern noted. Fowls whose erythrocytes gave a strong agglutination in the presence of the virus were selected and retained for periodic bleeding as a source of susceptible erythrocytes.

(U) Fowls were bled from the heart with a 20-ml syringe fitted with a three- to four-inch 19-gauge needle. Approximately 20 ml of blood was withdrawn every two weeks as needed and mixed with an equal volume of a two per cent sodium citrate solution to prevent clotting. The blood was stored at 4°C, used for one week and the remainder discarded.

(U) In preparing an erythrocyte suspension, blood cells in the citrated solution were centrifuged in an angle-head centrifuge at 1000 revolutions per minute (rpm) for ten minutes. The packed cells were washed with physiological saline, and centrifuged at 1000 rpm for ten minutes. The cells were washed three times in this manner. After the third washing, the cells were packed in a graduated tube by centrifugation at 1500 rpm for ten minutes, then diluted with saline to a one per cent erythrocyte suspension.

(U) The hemagglutination (HA) titer of a virus suspension was determined by placing 0.25 ml of saline in each of 10 or more tubes. A 0.25-ml suspension of undiluted virus was added to the first tube. A series of two-fold dilutions was made by mixing the contents of the first tube and passing 0.25 ml of the mixture to the second tube. This continued through the series of tubes with 0.25 ml discarded from the tenth tube. Then 0.25 ml of the one per cent chicken erythrocyte suspension was added to each tube, followed by 0.5 ml of saline. The final virus dilution in the first tube was 1:8. An erythrocyte control consisted of 0.25 ml of erythrocytes and 0.75 ml of saline. The total volume in each tube was 1.0 ml. All tubes were then vigorously shaken, incubated at 37°C for one hour and the cell patterns read at the bottom of the tubes. Agglutination was indicated by a ragged, irregular settling of cells. Negative tubes showed a compact, smooth "button." The HA titer of a virus suspension was recorded as the highest dilution of virus giving complete agglutination.

(U) In the hemagglutination-inhibition test, the virus suspension was diluted to contain four HA units per tube in the final dilution. This was termed the working virus suspension. If the HA titer of a virus suspension was 1:64, then four HA units were obtained by diluting the virus 1:16. All test sera were inactivated by heating at 56°C for 30 minutes. Both preinfection and postinfection sera from exposed animals were used to determine the H-I titer.

(U) The test procedure provided that 0.25 ml of saline was placed in each of ten or more tubes, then 0.25 ml of serum was added to the first tube. Serial twofold dilutions were subsequently made by passing 0.25 ml of the mixture from the first tube to the second. This was continued throughout the remaining tubes with 0.25 ml discarded from the last tube. The working virus suspension (0.25 ml) was added to each tube and mixed. The tubes were then incubated at 37°C for one hour. After this preliminary incubation period, 0.25 ml of a one per cent erythrocyte suspension was added to each tube, followed by 0.25 ml of saline. The tubes were well shaken, incubated at 37°C for one hour, and then read. The H-I titer of the sera was expressed as the highest initial dilution (i. e., the dilution before addition of antigen, erythrocytes and saline) which effected complete inhibition of agglutination. A fourfold rise in H-I titer over that of the preinfection sera was considered significant. Suitable controls, essential to the test, included the use of known positive and negative sera, erythrocyte suspensions, and virus dilutions.

2. (U) Serum Neutralization Test

(U) The serum neutralization test determines the presence of a specific neutralizing antibody and is generally a good index of immunity, since the serum antibody neutralizes active virus and prevents the initiation of infection. It is an in vivo procedure as compared with the hemagglutination-inhibition test, which is carried out in vitro. In this neutralization test, the strength of a serum required to neutralize the virus was measured by the ability of the serum to reduce significantly or to prevent pock formation by the agent on the CAM. Difficulties in the quantitation and interpretation of serum-neutralizing titers generally arise from the lack of statistical treatment of pock counts. Therefore, the limits of reproducibility of virus titrations are essential and should be determined prior to testing of the sera.

(U) Both preinfection and postinfection sera from inoculated or exposed animals were heated at 56°C for 30 minutes before testing. The test consisted of mixing equal volumes of known infectious virus units with increasing dilutions of sera. The virus dilution selected, when added to twofold dilutions of sera, gave approximately 50 infectious units per 0.05 milliliter of inoculum. Other quantities of virus can be used, as the neutralization potential of a serum obeys the percentage law. [15] Controls consisted of known positive and negative sera and titrations of virus. The mixtures

of virus and sera were incubated at 26° to 28°C for one hour prior to inoculation. Aliquots from each tube (0.05 ml) were inoculated on the CAM of 11- to 13-day-old embryonated eggs using seven or eight eggs per dilution. The eggs were then incubated at 35°C for 72 hours and pocks counted in the usual manner.

(U) Since the confidence limits of virus titrations about a mean were ± 0.3 log at the 95 per cent level, a 50 per cent reduction of pocks about the mean was considered significant. The serum-neutralizing titer was expressed as that dilution of serum which significantly reduced the number of pocks. A fourfold increase in the neutralizing titer of a post-infection serum over that of a preinfection serum was considered significant. Results of a sample SN test are recorded in Table IV.

TABLE IV. (U) REDUCTION OF VARIOLA POCKS ON CAM BY IMMUNE RABBIT SERUM

Serum Neutralization Test

Serum	Final Serum Dilution	Pocks per Membrane of Individual Eggs	Total	Avg.	Reduction from Mean, %	SN Titer
Normal Serum	1:2	3 21 22 40 30 45 42 22	225	28.1	21.3	
	1:8	14 30 25 14 5 35	123	20.6	42.3	7½
	1:16	24 3 11 43 27 33 15 71	226	28.2	21.1	
Immune Serum	1:2	4 0 5 2 0 0 0 0	12	1.7	95.3	
	1:8	6 3 0 1 4 1 6 5	26	3.2	91.1	
	1:16	4 0 0 3 9 9 11 7	43	5.3	85.2	1/32
	1:32	2 6 6 6 7 9	36	6.0	83.2	
	1:64	30 15 17 46 40 50 25	223	30.8	13.8	
Virus Control		34 35 28 13 1 20 55 80	274	34.2		
		39 39 36 60 20 16 63 26	299	37.3	Mean	
		18 12 38 18 35 33 28 105	287	35.8	35.7	

K. (U) VIRUS GROWTH CURVE TECHNIQUES

(U) The viral growth on the chorioallantoic membrane was estimated at different incubation temperatures and with varying doses of inoculum. Eggs were routinely incubated at 35°C unless indicated otherwise. Three to five chorioallantoic membranes were harvested at different intervals of time after inoculation to measure viral multiplication. Membranes harvested in the first eight hours after inoculation were washed two or three times in heart infusion broth to remove residual viral inoculum which could interfere with the determination of the actual amount of virus in membranes. The membranes were pooled and stored at -60°C. At the completion of a growth experiment, they were made into 20 per cent suspensions and assayed for viral content.

(U) A similar procedure was employed in studies to determine the viral content of the chorioallantoic membrane as a result of varying the volume of inoculum and the age of eggs.

L. (U) VIRUS DISTRIBUTION IN EMBRYONATED EGGS

(U) Groups of embryonated eggs eleven days old were inoculated with 5×10^3 infectious units in a volume of 0.05 milliliter by one of four routes (chorioallantoic membrane, allantoic, amniotic, or yolk sac) and incubated at 35°C for 49 hours. The following components were then harvested from each inoculated egg group: chorioallantoic membrane, embryo, yolk sac, yolk fluid, albumin, allantoic fluid, and amniotic fluid. The harvested parts from three to five infected eggs were pooled. Membranes and embryos were thoroughly rinsed in sterile heart infusion broth to reduce virus contamination from surrounding fluids. Tissues and albumin were made into ten per cent suspensions for titration; egg fluids were considered undiluted. The various components were assayed for virus. Several normal 13-day-old embryonated eggs, comparable in age to the virus-inoculated eggs after incubation, were selected at random and carefully dissected for removal of embryo, membranes, fluids, and albumin. The wet weight of these components was determined, and a mean value was obtained for each tissue or fluid (Table V). The total virus content of each tested component was calculated by multiplying the infectious units per milliliter or gram by the normal mean weight of each component.

M. (U) TISSUE CULTURE METHODS

1. (U) Cell Cultures

(U) Table VI shows the cell strains tested with virus and the media in which they were grown or supplied. Details of their morphology and cultivation by investigators responsible for their isolation can be found in the

accompanying references. The majority of cell strains were obtained from Microbiological Associates, Inc., as roller tube cultures containing 60,000 cells per tube, with the exception of Maben, L, and dog kidney epithelium strains. Both Maben and L cells were furnished by Dr. Arthur Brown, BW Laboratories. Cultures of Maben and L cells grown in Roux bottles were removed from the glass surface by treatment with 15 ml of Bacto-trypsin, 1:250, as a 0.5 per cent suspension in phosphate-buffered saline and were incubated at 37°C for 90 minutes. On occasion, cell growth was scraped into a small amount of mixture 199 and dispersed by pipetting. Cell suspensions were sedimented by centrifugation at 1000 rpm for five minutes in an angle-head centrifuge and resuspended in appropriate growth media (Table VI). After counting the cells in a hemocytometer the volumes of the suspensions were adjusted to yield the desired concentrations. Tubes were inoculated with approximately 100,000 cells and incubated at 37°C for three to four days in a stationary position. Dog kidney cell suspensions were prepared from excised kidneys of animals three to four months old by the method of Younger. [17] These cells were distributed with 100,000 cells per tube and cultivated in a manner similar to the procedure just described for Maben and L cell strains. Culture tubes in which cell inocula had formed confluent cell sheets were repeatedly rinsed with mixture 199 to remove growth media. Maintenance medium consisting of 10 per cent horse serum, 90 per cent mixture 199, 400 units per ml of penicillin and 100 micrograms per milliliter of streptomycin was added in two-milliliter amounts to all cultures just prior to virus inoculation.

2. (U) Techniques for Related Tissue Culture and Virus Studies

(U) The ability of the virus to induce a cytopathogenic effect in different cell strains was determined as follows: serial tenfold virus dilutions were made in maintenance media and 0.1 ml of each dilution was inoculated into three prepared tissue culture tubes. Control cultures were inoculated with similar dilutions of a 20 per cent CAM suspension prepared from normal 13-day-old embryonated eggs. Tubes were incubated at 35°C in roller drums turning at 12 revolutions per hour and examined daily for characteristic cytologic changes. Titers were expressed as number of tissue culture infectious doses ($TCID_{50}$) per 0.1 ml after three days of incubation, as calculated by the Reed and Meunch method. [18] The specificity of cytopathogenic effect in virus-inoculated cell strains was determined by a qualitative serum-neutralization test. For this a 10^{-1} virus dilution was mixed with an equal volume of normal or specific-immune rabbit serum. After incubation at 25°C for one hour, 0.1 milliliter of each mixture was inoculated into each of three culture tubes which were incubated as described previously and observed daily. Evidence of viral multiplication in cell strains was obtained by (a) serial dilution and passage of inoculum in several generations of cultures, or (b) growth curve experiments. Using procedure (a), 0.1 milliliter of a 10^{-4} virus dilution was inoculated into three or more culture tubes containing 1.9 ml of maintenance media, thus giving an initial virus dilution of $10^{-5.3}$. The media from inoculated tubes

were replaced every two or three days until cultures showed signs of degeneration. This procedure caused a further dilution of the original inoculum. Inoculum derived from the last change was then passed into fresh cultures. Media changes were stored at -60°C in an electrically operated freezer until assayed for infectivity. Viral growth curve experiments were performed by inoculating 0.1 milliliter of virus preparation diluted 10^{-1} or 10^{-3} into groups of cultures which were incubated at 35°C in roller drums. Sufficient cultures were inoculated so that the fluid contents of three replicate tubes were pooled daily for approximately ten days. The fluids were stored at -60°C until assayed for virus content.

TABLE V. (U) COMPONENT WEIGHTS OF NORMAL 13-DAY-OLD EMBRYONATED EGGS

Egg Component	Wet Weight, grams			
	Egg 1	Egg 2	Egg 3	Mean
CAM	0.8	0.9	0.6	0.7
Embryo	7.0	7.7	6.7	7.1
Allantoic Fluid	8.4	6.8	8.0	7.7
Amniotic Fluid	3.2	3.1	2.2	2.8
Yolk Sac	1.1	0.9	0.9	0.9
Yolk Fluid	12.9	12.9	7.1	10.9
Albumin	11.6	11.8	10.6	11.3

TABLE VI. (U) ORIGIN OF CELL STRAINS

CELL STRAIN	TISSUE SOURCE	GROWTH MEDIA	REFERENCE [f]
HeLa	Human carcinoma of cervix	$HuS_{10}BME_{90}$ [a]	19
KB	Human carcinoma of nasopharynx	$HuS_{10}BME_{90}$	20
Intestine	Jejunum from normal human embryo	$HuS_{10}BME_{90}$	21
Detroit-6	Sternal marrow from human with carcinoma of lung	$HuS_{10}BME_{90}$	22
Detroit-98	Normal human sternal marrow	$HuS_{10}BME_{90}$	22
Detroit-116P	Pleural fluid from human with lymphosarcoma	$HuS_{10}BME_{90}$	22
J-111	Blood cell from human with monocytic leukemia	$HuS_{10}BME_{90}$	23
D-189	Human infant foreskin	$HuS_{10}BME_{90}$	24
Conjunctiva	Normal human bulbar conjunctiva	$HuS_{20}BME_{80}$	25
Liver	Normal human liver	$HuS_{20}BME_{80}$	25
MK	Monkey kidney epithelium	$CS_2LH_{0.5}BSS_{97.5}$ [b]	17
DK	Dog kidney epithelium	$HoS_{10}LH_{10}BSS_{80}$	17
BK	Embryonic bovine kidney epithelium	$CS_{10}BME_{90}$	[e]
BM	Embryonic bovine skin and muscle	$CS_{10}BME_{90}$	[e]
S-180	Mouse sarcoma	$HoS_{10}BME_{90}$ [c]	26
L	Normal connective tissue from C_3H strain mouse	$HoS_{20}199_{80}$ [d]	27
Maben	Pleural fluid from human with carcinoma of lung	$HoS_{20}199_{80}$	28

a. 10% human serum, 90% basal medium (Eagle).
b. 2% calf serum, 0.5% lactalbumin hydrolyzate, 97.5% balanced salt solution (Earle).
c. 10% horse serum, 90% basal medium (Eagle).
d. 20% horse serum, 80% mixture 199.
e. Isolated by laboratories of Microbiological Associates, Inc., unpublished.
f. See Literature Cited.

III. (S) EXPERIMENTAL WORK

A. (C) FACTORS IN PROLIFERATION OF VARIOLA VIRUS IN EMBRYONATED EGGS

(U) This phase of study defined the characteristics of virus in embryonated eggs and the optimal conditions for obtaining preparations of high virus content.

1. (U) Growth Curves at Different Incubation Temperatures

(U) In the course of producing viral suspensions, inconsistent virus yields were often obtained from infected membranes after inoculation with a standard virus dose. This result was believed to be related to the incubation temperature, since the incubators in which inoculated eggs were held often fluctuated from the desired temperature. To evaluate this factor, a growth curve study was made of CAM-inoculated eggs incubated at different temperatures.

(U) Results plotted in Figure 15 revealed that the growth of variola in the chorioallantoic membrane varied sharply with the temperature. It was evident that 35°C was more conducive for viral multiplication than were other temperatures tested. This incubation temperature resulted in a short, ill-defined latent period prior to viral multiplication. The peak growth level was reached at approximately 46 hours. At 37°C, an extended latent phase was observed which persisted for 41 hours after inoculation. Maximum viral growth occurred in 49 hours and was 1.9 log lower in titer than the peak level found at 35°C. A five-hour latent period occurred at 39°C followed by a period of limited viral propagation during the ensuing 25 hours. The level of multiplication was only slightly above that found in the latent period. It then progressively declined until viral infectivity could no longer be demonstrated 65 hours after inoculation.

2. (U) Growth Curves with Different Doses of Inoculum

(U) The length of the latent periods noted in the three multiplication curves (Figure 16) varied inversely with the strength of the inoculum. A latent period (eclipse) of 11 hours' duration occurred with the smallest dose, 1.9×10^2 infectious units. It was a period in which no viral activity could be detected. The peak growth level was reached in 48 hours irrespective of the virus quantity initially inoculated. However, the two larger inocula, 1.9×10^6 and 5×10^3 infectious units, gave peak virus titers higher than that found with 1.9×10^2 infectious units of inoculum. The maximum rate of virus growth was independent of the virus quantity inoculated, as has been noted with other viruses. [29,30] The rate of increase is plotted against time in Figure 17 as log x/y where x is the amount of virus recovered and y is the amount of virus inoculated.

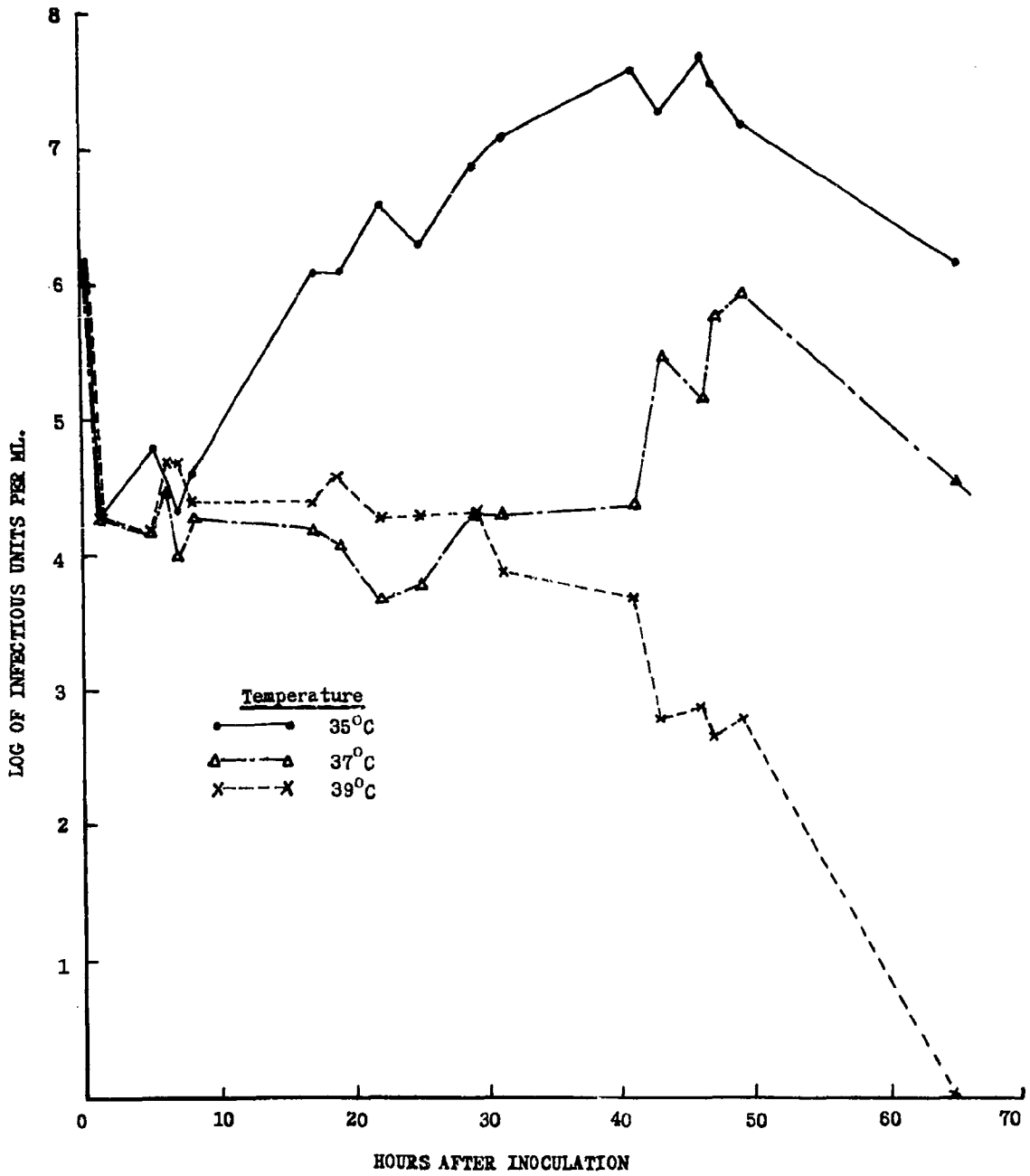


FIGURE 15. (U) GROWTH CURVE OF YAMADA STRAIN AFTER CAM INOCULATION OF 12-DAY-OLD EMBRYONATED EGGS INCUBATED AT DIFFERENT TEMPERATURES.

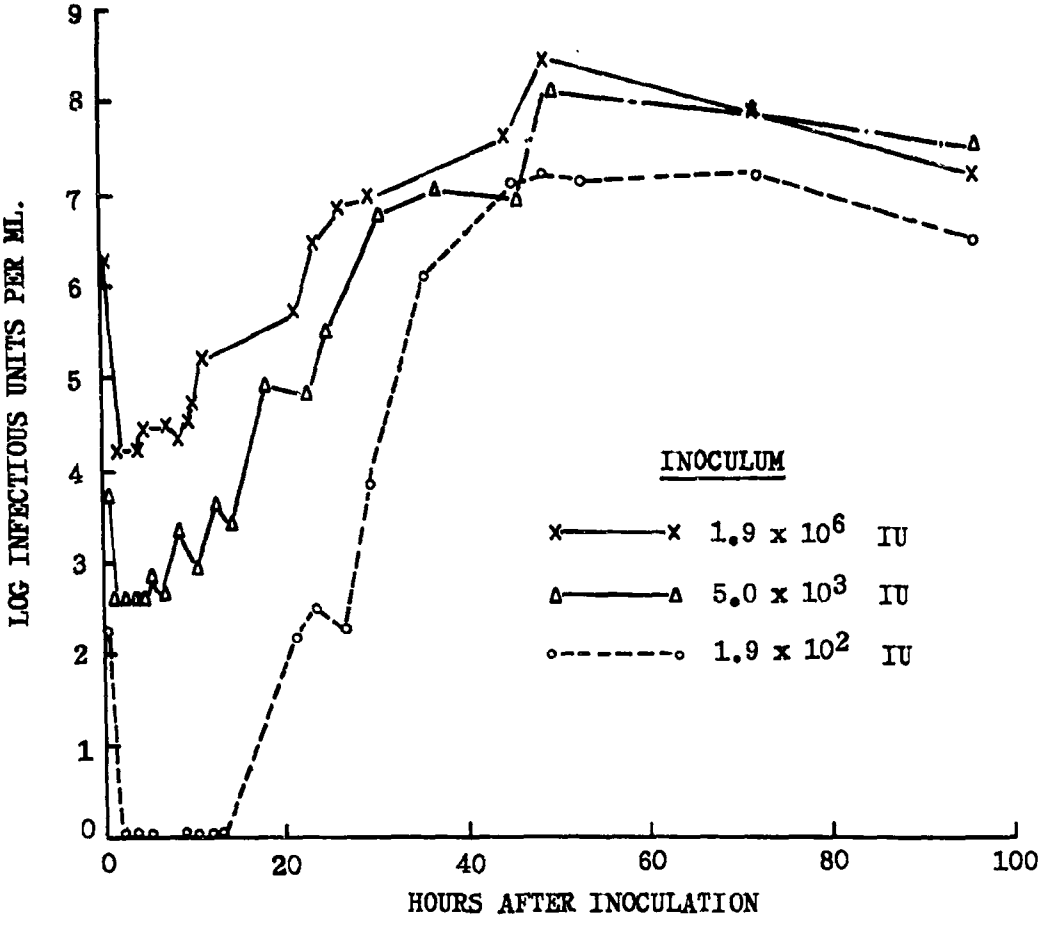


FIGURE 16. (U) GROWTH OF VARIOLA VIRUS ON CAM OF EMBRYONATED EGGS WITH VARIED DOSES OF INOCULUM.

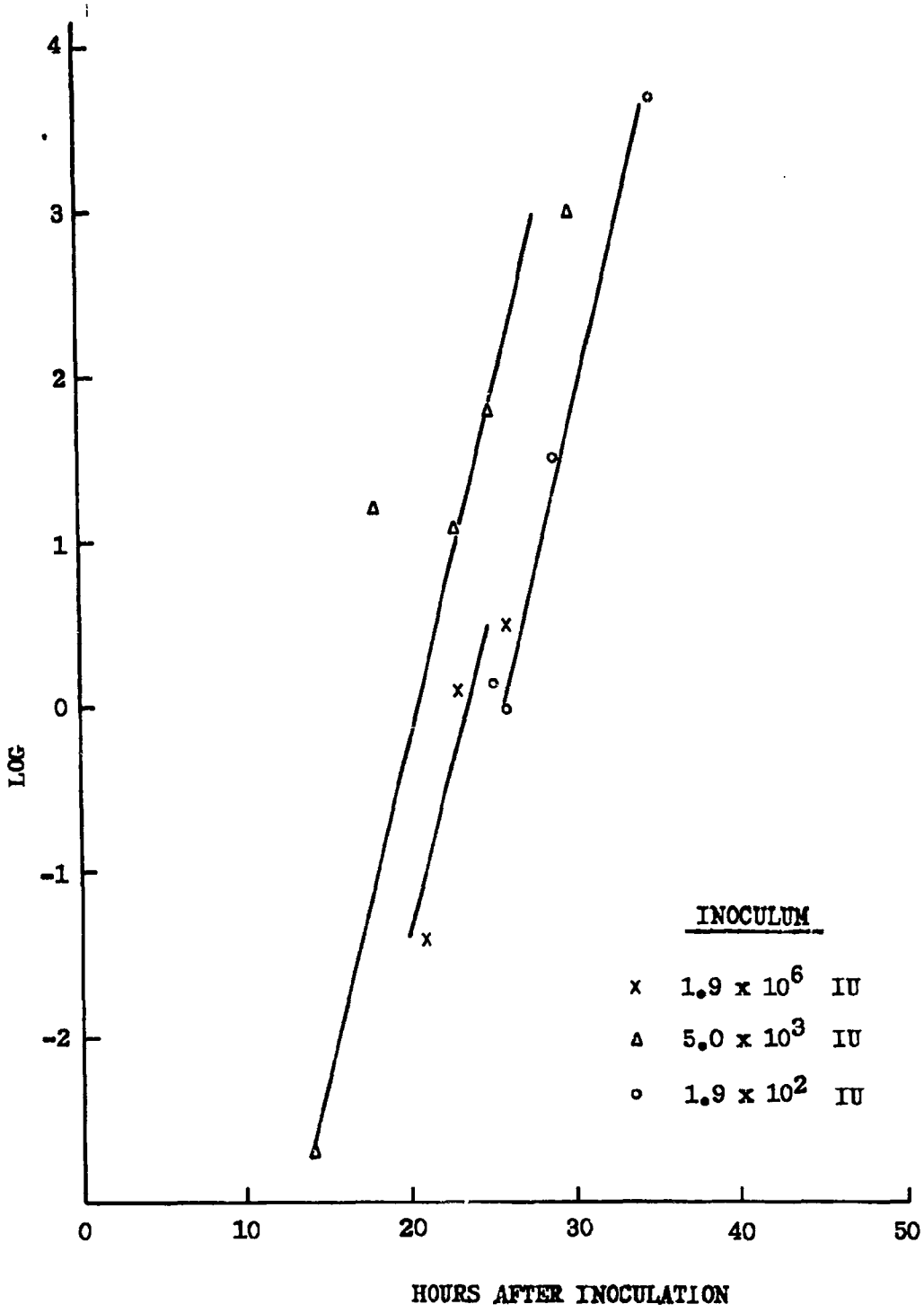


FIGURE 17. (U) LINEAR RELATIONSHIP BETWEEN AMOUNT OF VIRUS RECOVERED AND QUANTITIES OF VARIOLA VIRUS INOCULATED DURING PERIOD OF MAXIMUM VIRUS GROWTH.

Experimental points were plotted during the period of the highest rate of virus multiplication but not after limiting concentrations of virus were attained. Points for all three inocula formed straight lines with almost identical slopes. During this period, virus increased approximately 6000-fold per ten hours.

3. (U) Effect of Varying the Volume and Dose of Inoculum on Virus Yields

(U) The effect of increased inoculum volume on the virus content of membranes was investigated because it was conceivable that 0.05 milliliter was insufficient for disseminating virus to susceptible chorioallantoic membrane cells. Virus was diluted, therefore, so that the number of infectious units did not vary with different injected volumes. Results in Table VII indicate that the smallest volume, 0.05 milliliter, was capable of carrying virus to susceptible cells. Considering the variation inherent in titration, there was no apparent difference in the viral content of membranes inoculated with the varying volumes.

TABLE VII. (U) EFFECT OF VARYING THE VOLUME OF INOCULUM ON VARIOLA VIRUS YIELDS FROM INFECTED CAMS

VOLUME OF INOCULUM, ml[a]	YIELD[b] 10^8 IU per ml of CAM suspension
0.05	1.2
0.10	2.1
0.25	3.9
0.50	2.6

a. Each volume contained 5.0×10^4 IU.
b. After 48 hours.

(U) In the growth curve experiment (Figure 16) the peak viral content of the chorioallantoic membrane was influenced by the dose of inoculum. Because the doses tested were limited, it was of interest to test a more encompassing range. The results given in Table VIII show that the virus content of the chorioallantoic membrane was greatest with the three highest injected doses and progressively lower with those below 5.5×10^4 infectious units. The inoculation of 5.5×10^4 infectious units was the only dose that resulted in maximum virus content of the chorioallantoic membrane with a high rate of increase. Below this inoculum, a high rate of increase in virus occurred, but the peak virus content of membranes decreased in direct proportion to the dose.

TABLE VIII. (U) EFFECT OF VARIOUS DOSES OF VARIOLA INOCULUM ON VIRUS YIELDS FROM CAMS

DOSE, infectious units per 0.5 ml	VIRUS YIELD[a] FROM CAM	LOG INCREASE
5.5×10^6	2.6×10^8	1.7
5.5×10^5	1.8×10^8	2.5
5.5×10^4	2.1×10^8	3.6
5.5×10^3	1.8×10^7	3.5
5.5×10^2	3.6×10^6	3.7
5.5×10^1	5.6×10^5	4.0
5.5	3.0×10^4	3.8

a. Infectious units per ml after 48 hours.

4. (U) Virus Yield in Embryonated Eggs of Different Ages

(U) As shown in Table IX the age of eggs was an additional factor that influenced the maximum virus content of the chorioallantoic membrane. The quantity of virus was highest in embryonated eggs inoculated at nine to 13 days of age and correspondingly lower in eggs younger or older than this age span. The slight differences between virus quantities found in membranes in the group nine to 13 days old were approximately within the parameters of titration variability.

5. (U) Distribution of Virus in the Embryonated Egg

(U) Virus concentration in the various components of eggs inoculated by four different routes is shown in Figure 18. In those eggs inoculated by the chorioallantoic membrane route, the highest virus concentration was obtained from the chorioallantoic membrane and, in descending order, from the embryo, yolk sac, and other parts of the egg. The virus was disseminated to all the tested components. The total virus recovered, 1×10^7 infectious units, was indicative of multiplication, since it was significantly higher than the injected quantity of 5×10^3 infectious units. After amniotic inoculation, the highest concentration of virus was found in the embryo, with the chorioallantoic membrane containing the next lower amount. Viral multiplication occurred after amniotic inoculation because the total infectious units recovered, 1.9×10^4, was higher than the initial dose. Very little dissemination of virus was noted after allantoic inoculation, with no evidence of multiplication; virus recovery, 1.7×10^3 infectious units, was of a lower order than the amount injected. A moderate distribution was found after yolk sac inoculation with the greatest virus quantity appearing

TABLE IX. (U) VARIOLA VIRUS YIELDS FROM CAMS OF EMBRYONATED EGGS OF DIFFERENT AGES 48 HOURS AFTER INOCULATION[a]

AGE OF EMBRYONATED EGG, Days	YIELD, IU per ml
6	1.7×10^6
7	8.6×10^5
8	7.0×10^6
9	1.0×10^8
10	1.3×10^8
11	1.5×10^8
12	2.1×10^8
13	3.9×10^8
14	1.4×10^7
15	5.8×10^6

a. Inoculated with 5.5×10^4 infectious units.

in the yolk sac. At best, only limited propagation occurred after this route of injection. The total virus recovered, 9×10^3 infectious units, was only slightly above the injected quantity. The evidence supports the conclusion that the greatest distribution occurred after chorioallantoic membrane inoculation, with chorioallantoic membrane tissue providing the best growth medium.

6. (U) Influence of Biological Reagents on Virus Titer

a. (U) Cortisone Acetate

(U) An attempt was made to increase the virus yield from variola-infected membranes by treating embryonated eggs with cortisone acetate. This hormone has been reported to increase the multiplication of other viruses.[31,32]

(U) Cortisone acetate (Nutritional Biochemicals Corp., Cleveland, Ohio) was suspended in sterile distilled water and various amounts ranging from 0.5 to 5.0 milligrams were inoculated in the yolk sac of 11-day-old embryonated eggs. Groups of eight to ten eggs were inoculated with the hormone at four and 24 hours before virus inoculation, given the hormone simultaneously with virus inoculum, or injected at four and 24 hours after virus inoculation. Hormone-treated eggs were inoculated on the CAM with approximately 50 infectious units of virus and suitable diluent controls were similarly injected. Eggs were incubated at 35°C for 72 hours and examined for pocks. Since the variability of titrations differed about a mean by ± 50 per cent, increases or reductions greater than this were considered significant.

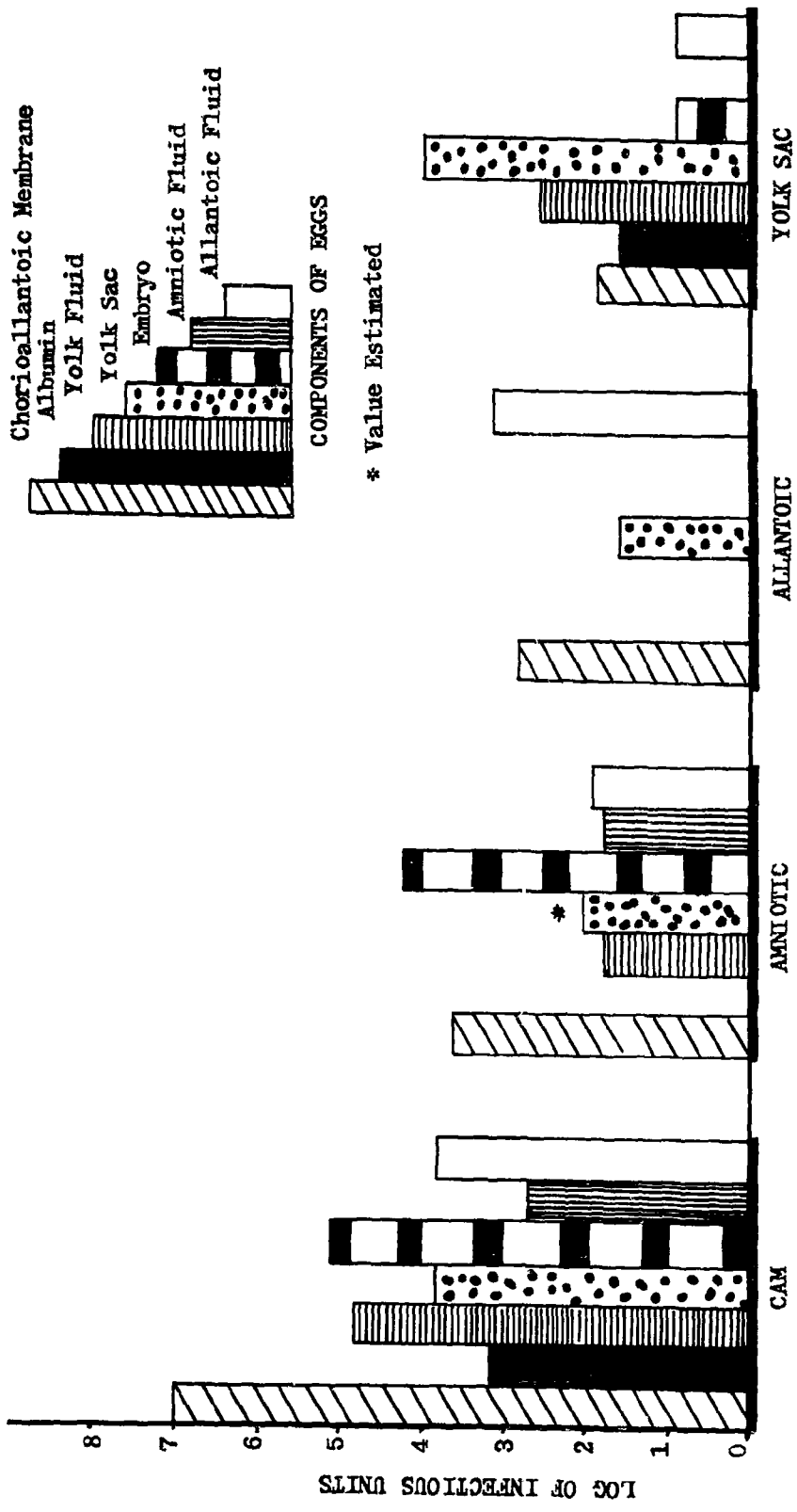


FIGURE 18. (U) DISTRIBUTION OF VARIOLA VIRUS (YAMADA) IN 13-DAY-OLD EMBRYONATED EGGS AFTER INOCULATION OF 5.0 X 10^3 IU BY VARIOUS ROUTES.

(U) Table X shows that a significant virus reduction of 94 per cent occurred in eggs treated with 5.0 milligrams of cortisone 24 hours prior to virus inoculation. Lesser quantities of the hormone administered at various time intervals revealed no significant change in virus titers, except where 0.5 milligram was inoculated 24 hours after virus inoculation. In this case a slightly higher titer of 83 per cent was noted but was similar to that of the control.

TABLE X. (U) INFLUENCE OF CORTISONE ACETATE ON VARIOLA VIRUS TITERS
Yolk sac inoculation of various doses of cortisone acetate before, with and after injection of virus on CAM

CORTISONE ACETATE, mg per 0.1 ml	EFFECT OF CORTISONE TREATMENT IN RELATION TO VIRUS INOCULATION AT 0 HOUR[a]				
	-24 hr	-4 hr	0 hr	+4 hr	+24 hr
5.0	-94	0	+45	-18	-27
2.5	-33	+24	-42	-25	+36
1.0	-19	+37	-19	-10	+56
0.5	+45	+24	-38	+18	+83
Diluent Control[b]	-38	+22	0	-51	+84

a. Per cent increase or decrease about a control mean of 53 infectious units.
b. Distilled water.

(U) Although not tabulated, the results of injecting cortisone via the allantoic sac in a similar test gave comparable results with no significant increase in virus titers. Under the conditions of these experiments variola virus titers in eggs were not increased as a result of cortisone treatment. In view of these findings, the effect of the hormone on virus yields from the CAM was not investigated.

b. (U) Hyaluronidase

(U) The enzyme hyaluronidase was tested in embryonated eggs to determine whether it would induce more extensive invasion of the agent on the CAM and thereby increase virus titers. Hyaluronidase is well known as a spreading factor which tends, in some instances, to increase the dissemination of certain infectious processes within animals.[33]

(U) Different concentrations of the enzyme (obtained from Mann Research Lab., Inc., New York, N.Y.) were inoculated in the yolk sac of 11-day-old embryonated eggs before, during and after CAM inoculation of virus. The enzyme in dried form, containing 150 turbidity reducing units per milligram, was reconstituted with sterile distilled water. The reconstituting fluid served as a control. Virus inoculum was diluted to give a sufficient number of pocks on membranes to be readily counted. This experiment was essentially identical with the previously recorded cortisone test. The results of the test are given in Table XI.

TABLE XI. (U) INFLUENCE OF HYALURONIDASE ON VARIOLA VIRUS TITERS
Yolk sac inoculation of varying doses of the enzyme before, with and after the injection of virus on the CAM

HYALURONIDASE, mg per 0.1 ml	EFFECT[a] OF HYALURONIDASE TREATMENT IN RELATION TO VIRUS INOCULATION AT 0 HOUR			
	-24 hr	-2 hr	0 hr	+24 hr
5.0	+30	+ 5	+17	-12
2.5	-18	0	+40	+27
1.0	0	+17	- 3	+ 8
Diluent Control[b]	- 6	0	+12	- 8

a. Per cent increase or decrease about a control mean of 135 infectious units.
b. Distilled water.

(U) Inspection of the data reveals no significant deviations of virus titers from the 50 per cent control mean. The enzyme appears not to affect virus titers by this method of testing.

(U) In a similar experiment, the enzyme was injected on the CAM of eggs before, during and after CAM inoculation with virus. This brought the enzyme into direct contact with susceptible cells and the virus inoculum. The purpose of this experiment was similar to that of the previous one; that is, to determine whether the enzyme can enhance the titer of the virus. The CAM was not treated with hyaluronidase 24 hours prior to virus inoculation, since it had been found previously that dropping the CAM 24 hours before virus inoculation caused a 1.0 log or greater reduction in virus titers. The concentrations of enzyme injection at various intervals, and the results, are shown in Table XII.

(U) The findings are similar to those of the previous test with the enzyme in that there was no significant enhancement of titers over that of the control mean. Hyaluronidase inoculated on the CAM apparently has no effect on virus titers under the conditions of this test.

TABLE XII. (U) INFLUENCE OF HYALURONIDASE ON VARIOLA VIRUS TITERS
CAM inoculation of varying doses of the enzyme before, with and after the injection of virus on the CAM

HYALURONIDASE, mg per 0.1 ml	EFFECT[a] OF HYALURONIDASE TREATMENT IN RELATION TO VIRUS INOCULATION AT 0 HOUR			
	-4 hr	0 hr	+4 hr	+24 hr
5	-47	-27	+23	-12
2.5	-28	- 5	+37	+ 3
1.0	-28	-19	- 4	-31
0.5	-30	-24	-23	+ 4
Diluent[b]	-29	+ 2	+57	-35

a. Per cent increase or decrease about a control mean of 88 infectious units.
b. Distilled water.

B. (U) SUSCEPTIBILITY OF VARIOLA VIRUS TO PHYSICAL AND CHEMICAL AGENTS

(U) The purpose of the following series of experiments was to determine the effect of physical treatments on the viability of the virus. The influence of chemical agents for disinfection and decontamination was also investigated.

1. (U) Effect of Ultrasonic Vibration on the Liberation of Virus from Infected Membranes

(U) The sonic oscillator is well known for its ability to liberate the contents of bacterial cells and cellular constituents of tissues. The current practice for making virus suspensions consists of disintegrating infected membranes in either a Ten Broeck tissue grinder or a Waring Blendor. The ultrasonic vibration treatment of infected membranes was compared with the Ten Broeck tissue grinding method. A number of infected membranes were divided into groups, each containing ten membranes and enough heart infusion broth to make a 20 per cent suspension. The suspensions were then subjected to ultrasonic vibration for a period varying from five to 30 minutes. For comparison, other membrane suspensions were disintegrated in a Ten Broeck tissue grinder. Ultrasonic vibration of membranes was accomplished in a Raytheon Sonic Oscillator, 15 amperes, 10,000 cycles per second, at 25°C. The treated suspensions were titered in the standard manner.

(U) There was no significant increase in virus liberated from CAMs by treatment with ultrasonic vibration for as long as 30 minutes. Virus titers were comparable to those obtained by Ten Broeck tissue grinding. A combination of both treatments gave titers similar to those obtained with the use of the Ten Broeck grinder alone.

2. (U) Effect of Repeated Freezing and Thawing on Viability of Liquid and Dried Virus

(U) Repeated freezing at -60°C and thawing at 28°C was tested for its effect on the viability of liquid and dried virus preparation. The liquid preparation was a 20 per cent concentration of infected membranes prepared in the usual way. A lyophilized virus suspension was used as the dried preparation. Samples of both preparations were repeatedly frozen and thawed. At designated intervals in the treatment, aliquots of each preparation were withdrawn and titered in the usual manner. The treatments and results on liquid and dried preparations are shown in Table XIII.

TABLE XIII. (C) EFFECT OF REPEATED FREEZING AND THAWING ON THE TITER OF VARIOLA VIRUS

NO. OF TESTS	REMAINING IU AFTER FREEZING AND THAWING CYCLES				
	0	1	3	5	10
			20 Per Cent Virus Suspension		
1	2.4×10^8 [a]	1.5×10^8	1.5×10^8	1.2×10^8	1.0×10^8
2	1.9×10^8	1.4×10^8	1.7×10^8	1.5×10^8	1.3×10^8
Mean	2.1×10^8	1.5×10^8	1.6×10^8	1.3×10^8	1.1×10^8
			Dried Virus		
1		1.8×10^9	2.7×10^{10}	3.2×10^{10}	2.5×10^{10}
2		4.7×10^9	2.0×10^{10}	1.5×10^{10}	3.2×10^{10}
Mean		3.1×10^9	2.3×10^{10}	2.3×10^{10}	2.4×10^{10}

a. IU per ml.
b. IU per gram.

(C) There was no difference between the initial titer of the liquid unfrozen preparation and that of the same preparation frozen and thawed for ten cycles. The 20 per cent suspension of virus appeared quite stable to the treatment. Stability of the dried virus compared favorably with that of the liquid preparation. The lower titer of the dried sample after the first cycle appears to be a technical error, as subsequent titers were uniformly higher.

3. (U) Effect of 56°C on 20 Per Cent CAM Suspension

(U) The inactivation of variola virus at 56°C was studied because this temperature is often used to inactivate serum complement prior to serological testing. Sealed ampoules containing 20 per cent virus suspensions were placed in a 56°C water bath for times ranging from ten to 120 minutes. At specified intervals the heated virus samples were withdrawn and immediately titered in eggs in the usual manner. The results are shown in Table XIV.

TABLE XIV. (U) EFFECT OF 56°C ON VARIOLA VIRUS IN A 20 PER CENT CAM SUSPENSION

TEST NO.	IU PER ML AFTER MINUTES OF TREATMENT		
	0	10	30
1	1.0×10^8	4.2×10^6	0
2	1.4×10^8	7.2×10^6	0
MEAN	1.2×10^8	5.7×10^6	0

(U) A reduction of 1.4 log in virus titer occurred during the first ten-minute treatment. No virus activity was detectable after the 30-minute treatment, resulting in a virus loss of 8.0 log. Longer treatment periods of 60 and 120 minutes also showed an absence of virus activity. The virus in a 20 per cent CAM suspension appears to be inactivated by 56°C for 30 minutes.

4. (U) Effect of pH on Virus Viability

(U) Virus viability was investigated in heart infusion broth (HIB) of varying pH levels, since this medium is used as a diluent and storage vehicle. It has been routinely used at pH 7.3.

(U) One milliliter of a virus suspension diluted 10^{-1} was added to nine-milliliter aliquots of HIB ranging in pH from 4.0 to 10.0. The HIB at pH 7.3 served as a control. Aliquots of each test medium held at 25°C were taken at four, 24, and 72 hours and titered on the CAM. The results are presented in Table XV.

TABLE XV. (U) EFFECT OF pH ON VIABILITY OF VARIOLA VIRUS AT 25°C

pH OF SAMPLE	HOURS OF STORAGE			
	0	4	24	72
4.0		2×10^2	2	2
5.0		2.5×10^5	2	2
6.0		1.6×10^6	2.5×10^3	2.0×10^3
7.3 (control)	1.5×10^6[a]	1.6×10^6	4.2×10^5	4.0×10^5
8.0		1.4×10^6	3.3×10^4	3.7×10^4
9.0		1.0×10^6	1.1×10^5	1.0×10^5
10.0		3.9×10^5	4.1×10^3	6.5×10^3

a. Infectious units per ml.

(U) The virus appeared to be equally viable in HIB, pH 6.0 to 9.0, when tested after four hours at 25°C. After 24 and 72 hours, pH 7.3 appeared best for maintaining virus viability. As with most viruses, the viability deteriorated more rapidly in acid than in alkaline diluents. Heart infusion broth, pH 7.3, was superior for maintaining virus viability at 25°C to the pH values below and above it.

5. (U) Effect of Chemical Agents on Virus Viability

a. (U) Lysol and Roccal

(U) An experiment was performed to determine the inactivating potential for the agent of the commonly used laboratory disinfectants, Lysol and Roccal. A 20 per cent suspension containing virus-infected membranes was diluted in equal volumes with two per cent Lysol and 400 ppm Roccal. The final virus concentration was 1.0×10^7 infectious units per milliliter and the final concentration of the chemical agents was one per cent Lysol and 200 ppm Roccal per milliliter. The control virus suspension was diluted in HIB. All test suspensions were held at room temperature (27°C). At specified intervals, aliquots from each test suspension were made into tenfold dilutions and titered on the CAM for viral activity. The results are shown in Table XVI.

(U) The data show that Lysol is an effective disinfectant for the agent. A final concentration of one per cent Lysol will inactivate 7.0 log of virus in approximately one minute. A 200 ppm concentration of Roccal was not as effective as Lysol, since virus viability was reduced only 5.0 log after five minutes of contact. In all probability a higher concentration of Roccal would be as effective as Lysol.

TABLE XVI. (U) EFFECT OF LYSOL AND ROCCAL ON THE VIABILITY OF 20 PER CENT SUSPENSIONS OF VARIOLA VIRUS AT 27°C

LENGTH OF EXPOSURE, minutes	INFECTIOUS UNITS PER ML			
	Control	1% Lysol	Control	200 ppm Roccal
1	2.0×10^7	0	1.0×10^7	1.0×10^7
5	1.6×10^7	0	1.0×10^7	1.0×10^2
15	1.4×10^7	0	0	0

b. (U) Ethyl Alcohol and Formalin

(U) The inactivating potentials of two other laboratory disinfectants were tested, ethyl alcohol and formalin. The testing of formalin was of particular interest because it was used in the procedure for examining infected CAMs. Two to three heavily infected membranes with confluent pocks were treated in toto with various concentrations of alcohol and formalin at 29°C for one hour, then pooled and thoroughly washed in saline. The membranes were homogenized, diluted and titrated in the usual manner. Controls consisted of infected membranes exposed to saline under identical conditions. The results are shown in Table XVII.

TABLE XVII. (U) INACTIVATION OF INFECTED MEMBRANES BY VARIOUS CONCENTRATIONS OF ETHYL ALCOHOL AND FORMALIN[a]

REAGENT TESTED	PER CENT CONCENTRATION OF REAGENT					
	1	2	10	50	70	95
Ethyl Alcohol			1.0×10^5[b]	1.9×10^2	4.9×10^2	7.6×10^1
Formalin	0	0	0	0		
Saline Control	1.0×10^8					

a. After one hour at 29°C.
b. Infectious units per milliliter.

(U) It is evident from the data that formalin was superior to ethyl alcohol in its inactivating potential under the conditions of testing. Concentrations of formalin as low as one per cent inactivated 8.0 log of the virus. Ethyl alcohol in 50 to 95 per cent concentrations reduced the

virus titer of treated membranes by approximately 6.0 log. However, virus activity was still present in all concentrations of the agent. Formalin is therefore considered suitable for inactivating infected CAMs prior to pock counting or for general decontamination procedures.

c. (U) Carboxide Gas

(U) Carboxide gas* is frequently used for decontaminating equipment or materials which would be damaged by heat sterilization or chemical disinfection. It was desirable therefore to determine its effect on variola virus.

(U) Infected membranes made into a 20 per cent suspension in HIB and a dried virus preparation were exposed separately in Petri dishes to Carboxide gas for 16 hours, 20 psi at room temperature (25°C) in an autoclave. Control samples were held at room temperature and atmospheric pressure. After treatment with the gas, both types of virus preparations were titered by the standard procedure. The results are shown in Table XVIII.

TABLE XVIII. (U) EFFECT OF CARBOXIDE GAS ON VARIOLA VIRUS IN A 20 PER CENT CAM SUSPENSION AND AS A DRIED PREPARATION

TEST NO.	TREATMENT	VIRUS SUSPENSION		DRIED VIRUS	
		IU per ml	log reduction	IU per gram	log reduction
1	Carboxide gas[a]	5.2×10^1	4.9	2.0×10^7	1.6
	Control	4.2×10^6		9.3×10^8	
2	Carboxide gas[a]	4.8×10^1	4.3	6.4×10^6	2.0
	Control	9.2×10^5		7.4×10^8	

a. For 16 hours at 25°C.

(U) The results show that the Carboxide gas significantly reduced virus activity under the conditions of the experiment. The dried preparation was the more resistant to inactivation. Treatment of virus-contaminated materials by Carboxide gas, under the conditions of this test, is not acceptable for decontamination. Exposure to the gas for longer periods and at higher temperatures may make it more effective.

* Ten per cent ethylene oxide and 90 per cent carbon dioxide.

C. (S) DRYING STUDIES

(C) Dehydration of the agent was investigated to determine its ability to withstand the process and to obtain a quantity of the dried material for stability and aerosol infectivity studies.

(S) Twenty per cent suspensions of infected membranes in HIB were made from 11- to 12-day-old embryonated eggs which had been inoculated on the CAM with approximately 5.0×10^3 infectious units per egg. The infected membranes were harvested after incubation at 35°C for 48 hours. The suspensions were lyophilized in Mylar trays. Suspensions were dried in 100- to 400-ml lots. The density was 1.01 with a mean solid content of 2.68 per cent. After lyophilization, aliquots of the dried preparations were accurately weighed and reconstituted with sterile distilled water to a volume that contained the same per cent solid content as did the original suspension. Each reconstituted suspension was serially diluted and titered on the CAM in the usual way. Approximately 20 virus suspensions were dried at various times during the course of the study. The results of four runs (Table XIX) show no significant loss in virus viability during the drying process. The loss of 7.2 per cent and 45.0 per cent are not significant, as these titers are in the range of 50 per cent variation. The titer of dried agent ranged from 1.0×10^9 to 4.1×10^9 infectious units per gram.

TABLE XIX. (S) EFFECT OF LYOPHILIZATION ON THE VIABILITY OF VARIOLA VIRUS

BATCH NO.	SOLIDS, per cent	PRE-DRYING TITER, IU per ml	POST-DRYING TITER, IU per ml	DRYING LOSS, per cent	TITER OF DRIED VIRUS, IU per gram
9	2.44	4.4×10^7	7.1×10^7	0.0	2.9×10^9
10	2.96	9.4×10^7	1.2×10^8	0.0	4.1×10^9
12	2.47	2.8×10^7	2.6×10^7	7.2	1.0×10^9
13	2.78	1.8×10^8	9.9×10^7	45.0	3.6×10^9

(C) On the basis of several trials variola virus appears to be resistant to lyophilization. The dried agent preparations were milled in the Tanner Spin Mill with no loss of virus potency. The resulting finely divided powder can be easily aerosolized.

D. (S) STORAGE STABILITY STUDIES

(C) Information on the storage stability of the agent, in both liquid and dried forms, was essential for evaluating its BW potential. Studies were initiated, therefore, to test the stability of the agent at various temperatures for specified periods of time.

1. (S) Stability of 20 Per Cent CAM Suspensions

(S) A 20 per cent CAM virus suspension, made in the customary manner, was distributed in two-milliliter amounts into glass ampoules and sealed with rubber stoppers at atmospheric pressure. Aliquots of the suspension were stored at -60°C (electric freezer), -25°, 4° and 25°C. Samples held at each storage temperature were periodically titered on the CAM of embryonated eggs. The stability throughout 64 weeks of storage is shown in Table XX.

(S) The liquid agent held at -60°C maintained its original potency after 64 weeks. At -25°C, the agent stability decreased slowly after two weeks through 64 weeks, but the loss was less than 1.0 log. The stability of the virus at 4°C for 16 weeks was approximately the same as that at -25°C. However, it declined in viability almost 3.0 log after 32 weeks and showed an over-all loss of 8.0 log after 64 weeks. Samples of the agent stored at 25°C showed a 3.0 log loss of titer in two weeks, and progressively declined until no activity could be detected after 16 weeks.

2. (S) Stability of Diluted Virus Suspensions

(U) The storage stability of diluted virus suspensions at -60°C was determined because impinger fluids of aerosol samplers are frequently stored at this temperature prior to titration in eggs.

(C) A working virus pool was diluted to concentrations of 10^{-3} and 10^{-5} in HIB. Several aliquots of each dilution were distributed into rubber-capped ampoules and stored at -60°C. Periodically, the potency of each stored dilution was determined by inoculation of the CAM in the usual way. Titers of aliquots stored as long as 191 days are shown in Table XXI.

(S) Virus dilutions of 10^{-3} stored for 54 days at -60°C did not significantly decrease in titer; the more dilute suspension of 10^{-5} showed a slight loss. However, after 85 and 191 days of storage, virus viability declined further although the loss in both dilutions was less than 1.0 log in magnitude. It would appear therefore that impinger fluids containing virus can be stored safely for 30 days without a significant loss of agent viability.

TABLE XX. (S) STORAGE STABILITY OF VARIOLA VIRUS IN 20 PER CENT CAM SUSPENSION

STORAGE weeks	NO. OF TITRATIONS	STORAGE TEMPERATURE			
		-60°C	-25°C	4°C	25°C
0	1	1.0×10^8 a/			
	2	9.7×10^7			
	3	1.4×10^8			
	Mean	1.1×10^8	1.1×10^8	1.1×10^8	1.1×10^8
2	1	1.2×10^8	1.4×10^8	5.7×10^7	2.0×10^5
	2	1.8×10^8	1.3×10^8	9.4×10^7	2.1×10^5
	Mean	1.5×10^8	1.3×10^8	7.5×10^7	2.0×10^5
4	1	1.1×10^8	7.2×10^7	5.2×10^7	2.7×10^5
	2	1.1×10^8	9.2×10^7	3.3×10^7	
	Mean	1.1×10^8	8.2×10^7	4.3×10^7	2.7×10^5
8	1	6.2×10^7	4.8×10^7	6.9×10^7	4
	2	7.4×10^7	7.4×10^7	9.4×10^7	
	Mean	6.8×10^7	6.1×10^7	8.1×10^7	4
16	1	1.5×10^8	3.9×10^7	1.4×10^7	0
	2	1.3×10^8	6.7×10^7	1.9×10^7	0
	Mean	1.4×10^8	5.3×10^7	1.6×10^7	0
32	1	2.3×10^8	6.2×10^7	4.8×10^5	
	2	1.5×10^8	4.6×10^7	3.0×10^5	
	Mean	1.9×10^8	5.4×10^7	3.9×10^5	
64	1	1.7×10^8	4.5×10^7	0	
	2	1.6×10^8	2.5×10^7	0	
	Mean	1.7×10^8	3.5×10^7	0	

a. Infectious units per ml.

TABLE XXI. (S) STORAGE STABILITY OF VARIOLA VIRUS IN DILUTE SUSPENSIONS AT -60°C

STORAGE, days	NUMBER OF TITRATIONS	STORED DILUTION	
		10^{-3}	10^{-5}
0	1	1.1×10^{5}[a]	6.1×10^{2}
	2	1.1×10^{5}	10.0×10^{2}
	Mean	1.1×10^{5}	8.0×10^{2}
18	1	1.0×10^{5}	3.8×10^{2}
	2	1.0×10^{5}	5.6×10^{2}
	Mean	1.0×10^{5}	4.7×10^{2}
54	1	11.0×10^{4}	8.0×10^{2}
	2	7.2×10^{4}	12.0×10^{2}
	Mean	9.1×10^{4}	10.0×10^{2}
85	1	4.2×10^{4}	7.8×10^{2}
	2	3.3×10^{4}	4.5×10^{2}
	Mean	3.7×10^{4}	6.1×10^{2}
191	1	6.0×10^{4}	3.4×10^{2}
	2	5.6×10^{4}	2.5×10^{2}
	Mean	5.8×10^{4}	2.8×10^{2}

a. Infectious units per ml.

3. (S) Stability of Dried Virus Preparations

(S) Storage stability at different temperatures was determined for a dried preparation which had been milled in the Tanner Spin Mill. The preparation was made by lyophilizing a 20 per cent CAM variola virus suspension which had a solid content of 2.68 per cent. The initial titer of the dried preparation was 3.8×10^9 infectious units per gram. It was dispensed in 0.5-gram quantities into rubber-capped ampoules under atmospheric conditions and stored at -60°, -25°, 4° and 25°C. At specified intervals, the samples held at each of the four temperatures were reconstituted with 4.5 milliliters of sterile distilled water to make a 10^{-1} dilution. Serial tenfold dilutions were made thereafter in HIB and appropriate dilutions titered on the CAM. The stability of the virus after 64 weeks of storage is shown in Table XXII.

(C) The dried preparation stored at -60° and -25°C for 64 weeks showed no significant loss of titer. A slight virus loss was noted when it was stored at 4°C after 32 weeks, but not at 16 weeks. The loss after 64 weeks was approximately 1.0 log. Storage at 25°C after two weeks showed a 1.0 log loss of titer which declined to a 4.0 log loss after 64 weeks.

E. (S) MONKEY INFECTIVITY STUDIES

(C) Variola virus is characterized by its inability to induce the typical disease in laboratory animals such as mice, guinea pigs, rabbits, and hamsters. Monkeys are reported to contract the disease naturally[34] and several species, Macaca rhesus, Macaca irus and others, have been infected with variola by the common routes of inoculation; the manifestation of the disease was somewhat similar to human infections.[35] Monkeys were used, therefore, as indicator hosts to test the infectivity of the virus.

1. (C) Parenteral Inoculation

(C) A preliminary experiment was used to familiarize laboratory personnel with the disease pattern induced by the virus in monkeys and to determine, qualitatively, the infectivity of the available strains.

(C) M. rhesus monkeys were inoculated parenterally with one of the seven available variola virus strains. The animals were inoculated both intradermally and intraperitoneally with approximately 1.0×10^7 infectious units by each route. As a control, a normal animal was caged with an inoculated one to observe the possibility of cross-infections between animals. The animals were kept under observation for 30 days.

TABLE XXII. (S) STORAGE STABILITY OF DRIED, MILLED VARIOLA VIRUS PREPARATIONS (S)

STORAGE, WEEKS	NO. OF TITRATIONS	STORAGE TEMPERATURE			
		-60°C	-25°C	4°C	25°C
0	1	4.4×10^9 [a]			
	2	4.7×10^9			
	3	2.4×10^9			
	Mean	3.8×10^9	3.8×10^9	3.8×10^9	3.8×10^9
2	1	2.0×10^9	1.5×10^9	6.4×10^8	17.5×10^7
	2	1.7×10^9	1.8×10^9	5.2×10^8	6.8×10^7
	Mean	1.8×10^9	1.6×10^9	5.8×10^8	1.2×10^8
4	1	1.3×10^9	1.7×10^9	2.1×10^9	9.1×10^7
	2	1.3×10^9		2.0×10^9	14.3×10^7
	Mean	1.3×10^9	1.7×10^9	3.5×10^9	1.1×10^8
8	1	3.3×10^9	2.0×10^9	2.9×10^9	5.0×10^6
	2	3.5×10^9	1.4×10^9	1.8×10^9	6.8×10^6
	Mean	3.4×10^9	1.7×10^9	2.3×10^9	5.9×10^6
16	1	1.9×10^9	1.2×10^9	1.7×10^9	1.1×10^6
	2	1.6×10^9	1.8×10^9	1.5×10^9	2.0×10^6
	Mean	1.7×10^9	1.5×10^9	1.6×10^9	1.5×10^6
32	1	1.9×10^9	1.8×10^9	7.1×10^8	5.9×10^5
	2	1.8×10^9	1.8×10^9	6.3×10^8	10.0×10^5
	Mean	1.8×10^9	1.8×10^9	6.7×10^8	7.9×10^5
64	1	1.2×10^9	1.9×10^9	5.9×10^8	0.69×10^5
	2	3.2×10^9	2.1×10^9	2.9×10^8	4.1×10^5
	Mean	2.2×10^9	2.0×10^9	4.4×10^8	2.3×10^5

a. Infectious units per gram.

(C) Four of the virus strains, Yamada, Lee, Stillwell and Harper, appeared to be equally infective for monkeys. Pustular eruptions of the skin were noted at approximately the eleventh day after inoculation and scabs, five to 10 centimeters in diameter were evident at the sites of intradermal inoculation. Two animals inoculated with the Gassman and Kim strains died early in the observational period. The specificity of these mortalities could not be determined. The Hartrige strain did not appear to infect the animal in which it was inoculated, as determined by gross observation. The control animal caged with an infected monkey appeared normal after the 30-day observational period.

2. (C) Intranasal Inoculation

(C) Another screening experiment was undertaken to determine the infectiousness of each virus strain for monkeys. Limited animal holding space required that the experiment be qualitative in scope. Six strains, Yamada, Lee, Harper, Stillwell, Kim, and Gassman, were used in the intranasal inoculation of M. rhesus monkeys. The animals were inoculated while under Nembutal anaesthesia, by instilling 0.5 ml of a 20 per cent CAM virus suspension into each nostril (a total of one milliliter per animal). Two animals were inoculated with each strain. The monkeys were observed daily for 26 days for overt signs of disease. The concentration of virus inocula, criteria of infection and results are shown in Table XXIII.

(C) All the virus strains were infective by the intranasal route of inoculation in the doses employed. Two of the animals that died never recovered from the effects of the Nembutal anaesthesia. The onset of pustules appeared between the eighth and tenth days after inoculation. A monkey infected with the Yamada strain with pustules on its face is shown in Figure 19. In all probability, less virus was required to infect the animal by intranasal instillation, as some of the inoculated material may have been swallowed and passed into the alimentary tract. Considering the qualitative nature of the method of inoculation and the number of animals involved, no distinction could be made as to which strain was more infectious.

3. (C) Oral Inoculation

(C) M. rhesus monkeys were inoculated orally with a stomach tube while under Nembutal anaesthesia. The following infectious units , contained in 10-ml doses, were introduced: 2.0×10^9, 2.0×10^4, and 2.0×10^2. Two animals were inoculated with each dose of virus. Temperatures of the animals were recorded twice daily for thirteen days and the animals kept under observation for an additional 15 days. The animals were bled at this time and a serum neutralization test was made to detect the antibody response.

TABLE XXIII. (C) INFECTIVITY OF VARIOLA VIRUS STRAINS IN M. RHESUS MONKEYS BY THE INTRANASAL ROUTE

VIRUS STRAIN	INOCULATED, infectious units	CRITERIA OF INFECTION: PRESENCE OF PUSTULES[a]
Harper	1.0×10^7	3
Harper	1.0×10^7	3
Stillwell	5.0×10^7	1
Stillwell	7.5×10^7	2
Yamada	3.0×10^7	Died - nonspecific
Yamada	3.0×10^7	1
Lee	2.8×10^7	1
Lee	2.8×10^7	Died - nonspecific
Kim	5.3×10^7	2
Kim	5.3×10^7	1
Gassman	5.0×10^6	3
Gassman	5.0×10^6	3

a. 3. Pustules on face, appendages and body trunk.
2. Pustules on face and appendages.
1. Three or more pustules on face.

FIGURE 19. (C) PUSTULAR LESIONS ON THE FACE OF A M. RHESUS MONKEY 12 DAYS AFTER DUAL INOCULATION WITH VARIOLA VIRUS (INTRAPERITONEAL AND INTRADERMAL). (C)

(C) The two monkeys receiving 2.0×10^9 infectious units showed elevations of temperature and significant serum neutralization titers. One animal of the two inoculated with 2.0×10^4 infectious units had an elevated temperature; however, sera from both animals showed no evidence of specific antibody. Two monkeys given 2.0×10^2 infectious units showed no fever or specific antibody as a result of inoculation. None of the inoculated animals exhibited any pustules. The two animals receiving 2.0×10^9 infectious units were considered infected on the basis of temperature elevations and antibody response. It would appear that M. rhesus monkeys are resistant to the agent introduced by oral inoculation. The disease in mild form was manifested only after high concentrations of the agent were employed.

4. (S) Aerosol Exposure

(S) Two species of monkeys, M. irus and M. rhesus, were exposed to an aerosolized 20 per cent CAM suspension of the Yamada strain. The purposes of the tests were (a) to determine whether monkeys could be infected by aerosolized virus, (b) to establish definitive criteria for diagnosing infection of monkeys with the agent, and (c) to compare the relative susceptibility of M. irus and M. rhesus monkeys.

(S) The two tests were made at 70°F and 50 per cent relative humidity. Eight milliliters of virus suspension containing 8.0×10^8 infectious units were disseminated with a Chicago atomizer. The monkeys were exposed for five-minute intervals to the aerosol at specified periods. The aerosol was sampled with all-glass impingers with flow rates of 12.5 liters of air per minute. The impingers contained ten milliliters of sterile HIB with one to two drops of olive oil. Duplicate impinger samples of the aerosol were taken. The impinger fluids were titered on the CAMs of 11- to 12-day-old embryonated eggs in the usual manner. The specificity of pustular eruptions was determined by scraping the pustules with a sterile knife blade and suspending the scrapings in HIB containing antibiotics. The suspension was inoculated on the CAMs of eggs for virus isolation.

(C) The M. irus monkeys ranged in weight from four pounds, four ounces to seven pounds, one ounce; the M. rhesus varied from three pounds, six ounces to five pounds. The animals were bled from the heart or femoral vein several days prior to exposure to secure pre-exposure sera. Rectal temperatures were taken twice daily for approximately two weeks after exposure. Control monkeys were caged with animals exposed to the test aerosol to detect cross-infections. Except for aerosol exposure, the controls were treated similarly to the test animals. All animals were observed for 30 days, after which they were again bled for postexposure sera.

(U) The diagnosis of any virus infection depends in varying degree on the establishment of four possible criteria: (a) a characteristic clinical picture, (b) isolation of the infecting virus, (c) a specific immunological response and (d) the presence in tissue of a pathological picture characteristic of that virus.

(U) In diagnosing smallpox in monkeys the following criteria were considered, (a) fever above 103.5°F, (b) vesicular or pustular eruption, (c) isolation of variola virus from pustules and (d) specific immunological response. It was arbitrarily decided that the fulfillment of two of the four criteria would constitute a variola infection in monkeys.

(U) The normal temperature mean of a quiescent monkey is 100.6°F. The temperature of normal, active monkeys ranges from 102.2°F to 103.5°F, with 104°F sometimes being normal. The specific fever pattern was defined as a temperature elevation of more than 103.5°F at approximately five to seven days after exposure. The isolation of virus was a confirmatory criterion since it depended on the presence of typical skin pocks. Pustules found between the eighth and eleventh days were considered specific for variola if virus was isolated from them. The immunological response was determined by measuring the neutralizing potency of the sera. A four-fold increase over that of normal sera was considered significant. The details of the test are given in Section II.

(U) The criterion for establishing infection by noting pathological tissue response characteristic of the virus was not considered. The histopathological procedure for preparing sections of pustular lesions routinely and staining for inclusions was time-consuming and impractical in that it involved animal sacrifice or surgery. Methods for staining of lesion scrapings for elementary bodies to establish the presence of virus are limited in reliability. The presence of elementary bodies is indicative of infection; however, their absence is considered inconclusive, and does not eliminate the possibility of infection. Since virus isolation from pustules by CAM inoculation of eggs is more reliable and serves the same purpose, no emphasis was placed on the staining of elementary bodies or inclusions as a criterion of infection.

(S) The pertinent data and results of the test with M. irus monkeys are shown in Table XXIV. The incubation period (time of exposure to onset of fever) was approximately four to six days, and therefore shorter than in human infections. It appeared to vary with the virus concentration inhaled, as the aerosol aged. The febrile response of the monkeys is shown in Figure 20. The febrile period was relatively short, lasting two to three days, with the fever peak occurring at the sixth day and declining on the seventh or eighth day. The animals suffered from malaise and loss of appetite during the period of fever elevation. Vesicles and pustules were observed eight to twelve days after exposure, remained for three days and then rapidly disappeared. The animals appeared normal thereafter.

TABLE XXIV. (S) INFECTIVITY OF M. IRUS MONKEYS EXPOSED TO AN AEROSOL OF VARIOLA VIRUS

INITIAL EXPOSURE AT minutes	RECOVERY, per cent	INHALED, IU	CRITERIA OF INFECTION[a]				DIAGNOSIS OF INFECTION
			Fever	Pustules	Virus Isolation	SN[b] Ab	
0 + 1	7.8	5.0×10^4	yes	2[a]	yes	no	yes
0 + 6	5.1	3.1×10^4	yes	0	no	no	no
0 + 15	5.0	3.4×10^4	yes	0	no	yes	yes
0 + 20	4.2	3.1×10^4	yes	0	no	no	no
0 + 30	4.3	2.7×10^4	yes	1	yes	yes	yes
0 + 35	2.3	1.6×10^4	Died				
0 + 60	0.9	7.4×10^3	yes	1	yes	yes	yes
0 + 65	1.7	1.3×10^4	yes	3	yes	yes	yes
Cage Control		0	no	0	no	no	no
Cage Control		0	no	0	no	no	no

a. 3. Pustules on face, appendages and body trunk.
2. Three or more pustules on face.
1. One or two pustules.

b. Serum-neutralizing antibody.

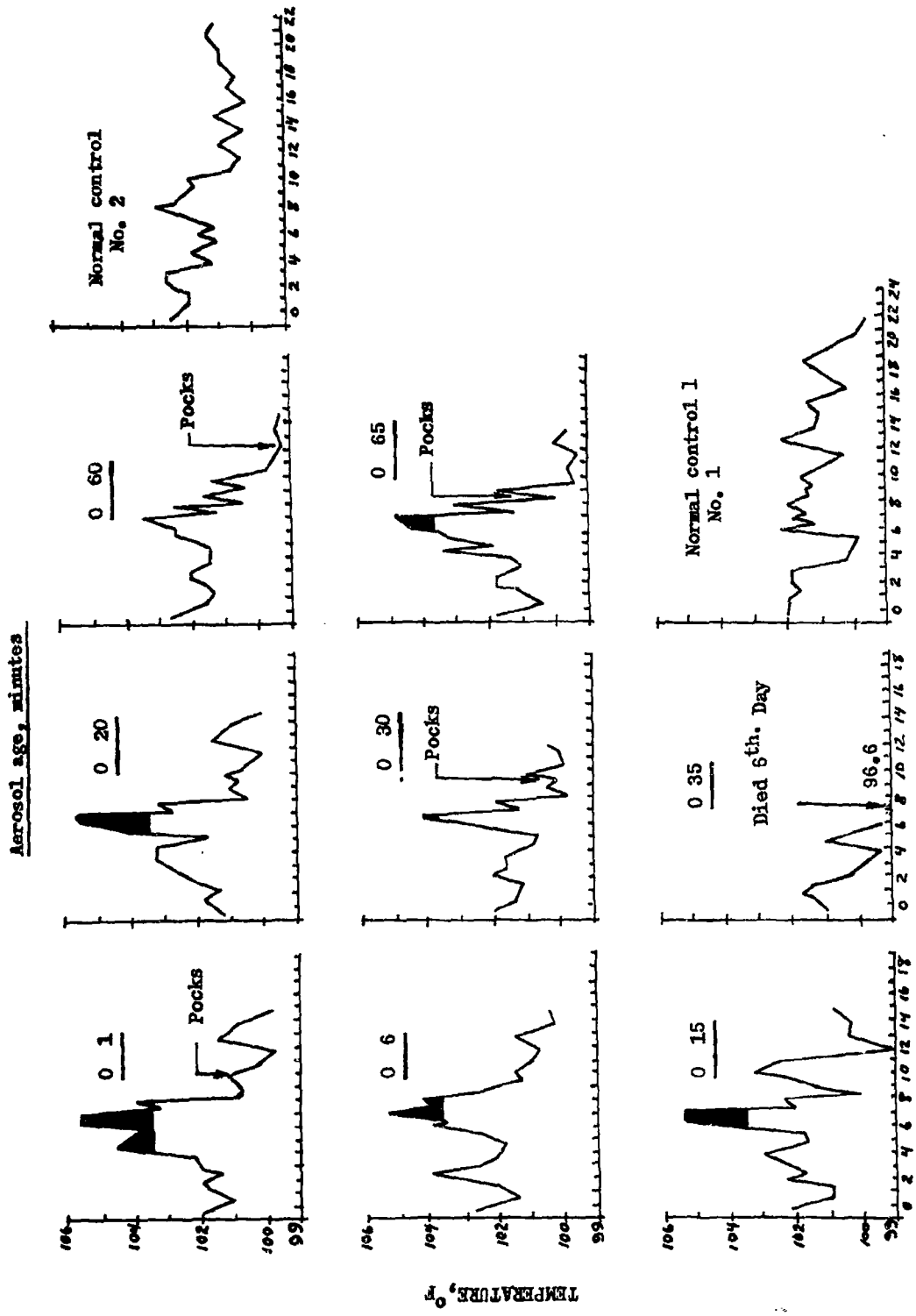


FIGURE 20. (C) FEVER RESPONSE OF M. IRUS MONKEYS EXPOSED TO AN AEROSOL OF VARIOLA VIRUS. (C)

None of the control animals showed signs of definitive fever. Exposed monkey 3912 developed a facial twitch and paralysis of the right arm, and died on the sixth day after exposure. The animal was autopsied and the only gross pathological condition noted was a cerebral edema in the left hemisphere. Although all exposed animals showed a febrile response, only five of the seven were considered infected according to the established criteria. Virus was isolated from all four animals exhibiting pustular lesions. Serum-neutralizing antibody was found only in four of the five infected monkeys and in three of the four monkeys showing pustular lesions. Normal control monkeys caged with infected animals showed no sign of disease. Apparently, cross-infection between caged animals was at least minimal.[34]

(S) The results of the test in which M. rhesus monkeys were exposed to the virus aerosol are summarized in Table XXV, with febrile responses depicted in Figure 21. Body temperatures greater than 103.5°F were noted in several of the exposed animals. The fever patterns were irregular, arising and persisting on different days. The fever response could not be accurately defined. The high degree of excitability of M. rhesus monkeys may account for the varied fever patterns. The concentrations of virus inhaled by the M. rhesus monkeys were almost identical with those inhaled by the M. irus in the first exposure intervals of an hour. The test with M. rhesus monkeys was different in that two animals were exposed to the aerosol after it had aged for as long as two hours. Infection was recorded, however, in a monkey exposed to an aerosol that had aged for two hours. Four of eight M. rhesus monkeys exposed to the aerosol were infected. The one normal control monkey was not infected when caged with an exposed monkey. A significant observation was the absence of pustules on the skin of M. rhesus animals, whereas four of the seven exposed M. irus monkeys exhibited some degree of pustular eruption.

(C) A neutralizing component was detected in normal inactivated sera from two of the three noninfected M. rhesus monkeys. This may account for the inability to infect some of these animals with the virus concentration inhaled. The apparent specificity of this normal serum from M. rhesus animals for the virus may possibly be due to exposure in an endemic smallpox area such as India, where these animals originate. M. irus monkeys come from the Philippine Islands, Malaya or the East Indies.

(S) The results of these two tests show the susceptibility of monkeys to aerosolized virus as disseminated in the SO Division exposure system. These tests suggest that M. irus monkeys may be a better indicator host of the disease than the M. rhesus. Additional experimental evidence is required for confirmation.

TABLE XXV. (S) INFECTIVITY OF M. RHESUS MONKEYS EXPOSED TO AN AEROSOL OF VARIOLA VIRUS

INITIAL EXPOSURE AT minutes	RECOVERY per cent	IHNALED IU	CRITERIA OF INFECTION[a] Fever	SN Ab[b]	INFECTIVITY
0 + 1	9.0	5.1×10^4	no	no	no
0 + 6	5.6	2.7×10^4	yes	yes	yes
0 + 30	3.3	1.7×10^4	no	yes	no
0 + 35	1.0	4.6×10^3	yes	yes	yes
0 + 60	0.9	3.8×10^3	no	no	no
0 + 65	2.4	1.2×10^4	yes	yes	yes
0 + 120	0.2	1.3×10^3	yes	no	no
0 + 125	0.1	3.5×10^2	yes	yes	yes
Cage Control		0	no	no	no

a. No pustules or virus isolations were detected in these tests.
b. Serum-neutralizing antibody.

F. (S) AEROSOL STUDIES

(S) It was necessary in aerosol infectivity studies to insure the maximum effectiveness of the agent by defining its aerosol characteristics, optimal conditions for dissemination, and methods of sampling. All aerosol studies with the agent were made in the 4800-liter test tank. The findings in this phase of investigation are only preliminary.

1. (S) Length of Aerosol Sampling Time on Virus Recovery

(S) In evaluating the aerosol potential of variala the effect of the length of the sampling period on recoveries was noted. A one-minute sampling period collected almost as much of the virus aerosol as did a five-minute sampling period. An experiment was designed to evaluate further this apparent finding.

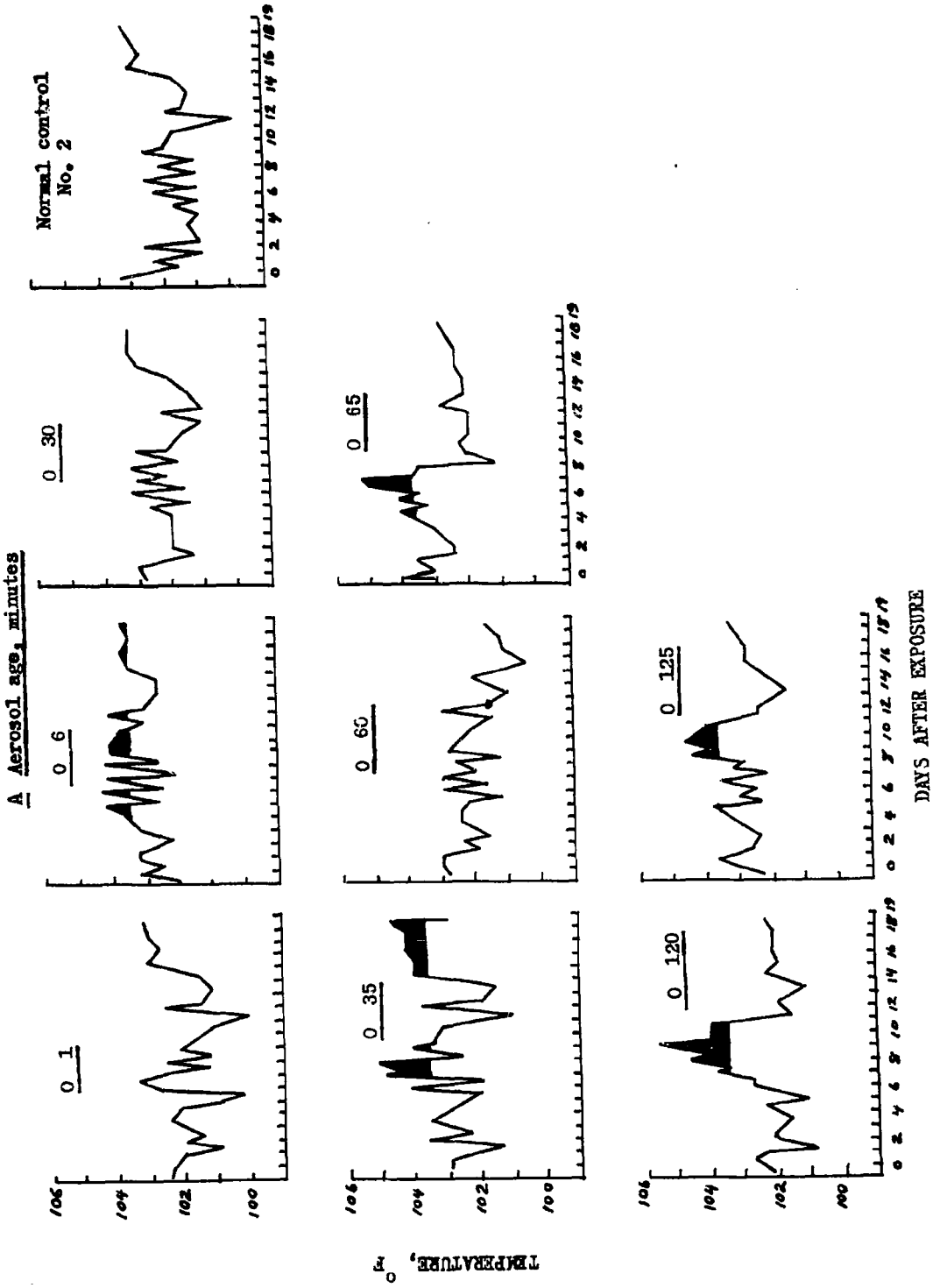


FIGURE 21. (C) FEVER RESPONSE OF M. RHESUS MONKEYS EXPOSED TO AN AEROSOL OF VARIOLA VIRUS. (C)

(S) In six separate tests the virus was aerosolized in the test tank and sampled for one and five minutes at cloud ages of 1.5 and 15.5 minutes. Eight milliliters of a 20 per cent CAM virus suspension was disseminated through an FK-8A atomizer into air at 70°F and 50 per cent relative humidity. This quantity contained 6.9×10^8 infectious units. Aerosol samples were collected at the rate of 12.5 liters per minute in all-glass impingers containing 20 ml of HIB. The impinger fluid was titered on the CAMs of eggs in the usual manner and by appropriate calculation the per cent recoveries were determined. The results are shown in Table XXVI.

TABLE XXVI. (S) EFFECT OF SAMPLING TIME ON PER CENT RECOVERIES OF VARIOLA VIRUS AEROSOLS

PER CENT RECOVERY OF AEROSOL SAMPLED FOR 1 MIN.		PER CENT RECOVERY OF AEROSOL SAMPLED FOR 5 MIN.	
Age of Aerosol Sampled			
1.5 min.	15.5 min.	1.5 min.	15.5 min.
23.3	41.4	12.2	10.6
22.2	13.3	14.4	8.4
41.4	20.0	14.4	6.6
18.9	5.5	10.6	8.9
55.6	8.9	33.3	17.8
72.0	15.5	13.3	11.1
Arith. Mean			
38.8	17.4	16.3	10.5

(S) The arithmetic mean of the tests showed a higher recovery of aerosolized virus in the first one-minute sampling period compared with the five-minute period. This higher recovery was evident in all the one-minute sample tests when the cloud was collected at 1.5 minutes' age. A fivefold increase in virus recovery was not evident when the cloud was collected for five minutes as compared with one minute. Apparently, some destruction or physical loss of virus occurred in the impinger which may be dependent on the collecting fluid or the impinger itself.

2. (S) Different Impinger Fluids on Virus Recovery

a. (S) Experiment 1

(S) A study was made on the effect of various collecting or impinger fluids on recovery of the aerosol. In each of eight different trials the cloud was sampled with the following impinger fluids: distilled water, heart infusion broth (HIB), ten per cent rabbit serum in saline (RSS) and one per cent D-sorbitol. Eight milliliters of a 20 per cent CAM virus suspension containing 6.9×10^8 infectious units were aerosolized in each test by a FK-8 atomizer at 70°F and 50 per cent relative humidity. The aerosols were sampled with all-glass impingers containing 20 ml of the different collecting fluids for five minutes at the following periods as the aerosol aged: 1.5 to 6.5, 15.5 to 20.5, 29.5 to 34.5 and 57.5 to 62.5 minutes. The collecting fluids were titered on the CAM of eggs (Table XXVII).

(S) The arithmetic means of eight replications with all impinger fluids at the various sampling intervals showed that ten per cent rabbit serum in saline consistently gave the highest recoveries from variola aerosols. The mean recovery with this impinger fluid was higher than with other tested fluids in the first sampling period and in subsequent sampling intervals.

(U) The mean decay rates of the aerosol tested with four different impinger fluids were similar, ranging from 4.3 to 6.9 per cent per minute over 56 minutes.

b. (S) Experiment 2

(S) Additional tests were made to observe the effect of different impinger fluids on recovery of variola aerosols. Twelve aerosols were disseminated and each cloud sampled with HIB, ten per cent rabbit serum in saline, 20 per cent yolk fluid and ten per cent skim milk. Eight milliliters of a 20 per cent CAM virus suspension containing 6.9×10^8 infectious units were aerosolized in all tests with a FK-8 atomizer at 70°F and 50 per cent relative humidity. All-glass impingers containing 20 ml of the different collecting fluids were used to sample the aerosol for one minute when the aerosol was two to three and 16 to 17 minutes old. The collecting fluids in each test were frozen at -60°C and later titered on the CAMs of eggs.

(C) From the results shown in Table XXVIII it is evident that both 20 per cent yolk fluid and ten per cent skim milk gave higher mean recoveries than HIB or ten per cent rabbit serum in saline. Although the yolk fluid gave a mean per cent recovery higher than that obtained with skim milk, the significance of this difference requires further evaluation. Additional tests are planned to determine the optimal means for sampling aerosols of this agent before studies are undertaken to characterize the virus cloud.

TABLE XXVII. (S) PER CENT RECOVERIES OF VARIOLA VIRUS AEROSOL WITH DIFFERENT IMPINGER FLUIDS

	AGE OF AEROSOL SAMPLED															
	1.5 to 6.5 min.				15.5 to 20.5 min.				29.5 to 34.5 min.				57.5 to 62.5 min.			
	Dist. Water	HIB	RSS	1% D-sorbitol	Dist. Water	HIB	RSS	1% D-sorbitol	Dist. Water	HIB	RSS	1% D-sorbitol	Dist. Water	HIB	RSS	1% D-sorbitol
	---	20.0	57.8	1.40	0.14	16.6	13.3	0.80	1.5	7.1	21.1	1.00	0.009	1.7	3.5	0.30
	0.70	16.6	35.6	4.40	0.35	1.1	15.5	0.80	0.01	0.5	11.1	0.30	0.10	1.0	1.8	0.01
	0.40	22.2	64.5	1.00	0.10	8.6	4.0	1.00	0.03	8.2	6.0	0.50	0.004	3.7	1.7	0.003
	0.40	11.1	---[a]	0.02	0.10	4.2	5.7	0.02	0.10	2.8	---	0.004	0.02	0.8	1.7	0.0006
	0.50	---	13.3	3.30	0.17	4.4	11.1	0.70	0.15	3.1	2.2	0.10	0.06	0.3	2.2	0.01
	0.40	7.3	---	6.00	0.18	4.2	---	1.00	0.04	1.1	3.5	0.10	0.06	0.7	2.1	0.004
	2.6	22.2	51.2	1.90	0.18	10.6	14.4	0.10	0.80	5.7	8.0	0.004	0.07	2.1	7.5	0.09
	1.7	22.2	60.1	7.10	0.05	2.6	17.8	0.50	0.04	5.7	13.3	0.02	0.03	2.1	4.4	0.001
Arith. Mean	0.90	17.5	47.0	3.10	0.07	6.5	11.7	0.61	0.33	4.2	9.3	0.25	0.04	1.5	3.1	0.05
Per cent Decay Rate per Min.													5.5	4.3	4.5	6.9

a. Not tested.

TABLE XXVIII. (S) PER CENT RECOVERIES OF VARIOLA VIRUS AEROSOL WITH DIFFERENT IMPINGER FLUIDS

AGE OF AEROSOL SAMPLED, minutes							
2 to 3				16 to 17			
HIB	RSS	20% Yolk Fluid	10% Skim Milk	HIB	RSS	20% Yolk Fluid	10% Skim Milk
15.4	20.0	56.7	65.7	21.4	5.4	43.1	38.9
9.0	21.1	40.0	69.0	7.8	15.5	17.8	23.3
8.3	8.9	40.6	38.6	15.5	14.4	26.7	55.6
24.7	22.2	66.7	30.0	3.6	3.0	12.8	29.4
12.2	6.3	52.3	47.8	5.2	1.5	20.0	15.0
25.3	25.0	77.9	34.7	18.9	10.6	17.8	18.9
9.9	12.1	89.0	72.3	9.2	6.3	32.2	26.7
13.3	36.7	55.6	46.1	7.2	12.2	43.4	7.8
10.5	35.0	94.6	61.2	8.9	19.7	30.0	34.5
13.9	38.9	51.7	40.6	7.7	3.1	17.8	5.5
9.4	15.0	46.7	48.3	3.5	10.0	16.1	20.0
27.2	46.7	63.3	83.4	10.0	18.9	29.4	42.4
Arith. Mean							
14.9	23.9	61.2	52.7	9.9	10.0	27.2	26.4

G. (C) TISSUE CULTURE STUDIES

(C) Tissue culture techniques contributing to recent developments in virology were applied to the study of variola virus. The principal objective of this study was to explore the possibility of using tissue culture methods as a research tool with this agent. The behavior of variola virus in tissue culture was investigated by using a number of human and animal cells derived from both normal and malignant tissues, since to our knowledge no information of this nature was available in the literature. Both the cytopathogenicity of the virus and its propagation in cells was studied.

1. (U) Cytopathogenicity

(U) All cell strains of human and animal origin derived from both malignant and normal tissue inoculated with variola virus showed cytologic changes except strain L cells (Table XXIX). In general, cellular changes consisted of rounding, swelling and clumping of cells with cytoplasmic granulation which were observed 24 to 48 hours after inoculation with concentrated virus inocula. Cell sheets became vacuolated, with subsequent degeneration and detachment from tube walls after incubation for 72 to 96 hours. Normal CAM suspensions did not produce distinctive cellular changes. Representative fields of uninoculated cultures and of infected cultures with degenerative changes are shown in Figures 22 through 35. These figures are photomicrographs of unstained normal and infected tissue culture fields showing the cytopathogenic effect of variola virus. They were photographed at 100X. Specific-immune serum but not normal serum completely prevented or delayed the cytopathogenic effect of virus in cell strains by 24 to 48 hours. Virus regularly induced cytologic changes after passage in four or five generations of the cell strains tested. Cytologic changes were produced by variola virus in 19 consecutive transfers from culture to culture in Maben cells. The total lapsed time in culture was approximately 95 days. Strain L cells were agglutinated by concentrated viral inoculum but not beyond the 10^{-1} dilution. Agglutination evidently required about 10^6 infectious units. Cell agglutination occurred within 24 hours and was inhibited by immune serum. A similar agglutination of L cells by vaccinia as demonstrated by Mayyasi et al[36] has been the basis of a rapid method for titration of vaccinia virus and its "neutralizing" antibody.

2. (U) Propagation

a. (U) Serial Passage

(U) Evidence of viral multiplication in cell strains was obtained by serial dilution and passage of inoculum in several generations of cultures. The following representative tables depict the method and evidence for the propagation of virus in HeLa cells (Table XXX), KB cells (Table XXXI), and L cells (Table XXXII). Results of cell strains tested in this manner are shown in summary form in Table XXXIII). It was evident that viral infectivity existed after the original virus inoculum of 2.2×10^3 infectious units per two-milliliter culture was sufficiently diluted to insure loss of infectivity had multiplication not occurred. Identical viral inoculum introduced in cell-free media was almost completely exhausted at dilution $10^{-7.9}$. The presence of virus in culture after prolonged incubation periods was additional evidence of multiplication. These periods exceeded the control by 12 to 21 days. Virus titers which were obtained after passage in many of the cell strains were greater than the original inoculum by 1.0 log or more. However, in some cultures, titers were below the initial virus amount inoculated. The limited quantities of virus produced from cultures were attributed to the rapidity of virus passage. Frequently, virus transfer was made after only two or three days of incubation, an interval too short to permit maximum viral multiplication. Subsequent data from growth curve experiments showed that peak viral titers occurred after incubation for approximately one week.

TABLE XXIX. (U) INITIAL CYTOPATHOGENICITY OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF HUMAN AND ANIMAL TISSUES

CELL CHAIN TESTED	HUMAN (H) OR ANIMAL (A)	NORMAL (N) OR MALIGNANT (M)	CYTOPATHOGENIC EFFECT 3 DAYS AFTER INOCULATION WITH VIRUS DILUTIONS				
			Undil.	10^{-1}	10^{-2}	10^{-3}	10^{-4}
HeLa	H	M	+++[a]	+++	+++	+++	+++
KB	H	M	+++	+++	+++	+++	??
Maben	H	M	+++	+++	+++	---	---
Liver	H	M	+++	+++	+++	---	---
Detroit-6	H	M	++	++	++	--	--
J-111	H	M	++	++	?	--	[b]
Detroit-116P	H	M	+++	+++	+++	+++	---
Intestine	H	N	+++	+++	?	--	--
D-189	H	N	+++	+++	+++	??-	---
Conjunctiva	H	N	+++	+++	+++	+?	---
Detroit-98	H	N	+++	+++	---	---	[b]
S-180	A	M	+++	+++	+++	???	?
L	A	M	AAA	AAA	---	---	---
Monkey Kidney	A	N	+++	+++	+?	---	---
Bovine Kidney	A	N	+++	+++	---	---	---
Dog Kidney	A	N	+++	+++	---	---	---
Bovine Muscle	A	N	+++	+++	+++	---	---

a. Each + indicates a complete cytopathogenic effect in one culture tube.
Each ? indicates a partial cytopathogenic effect in one culture tube.
Each - indicates no cytopathogenic effect in one culture tube.
Each A indicates agglutination of L cells.

b. Not tested.

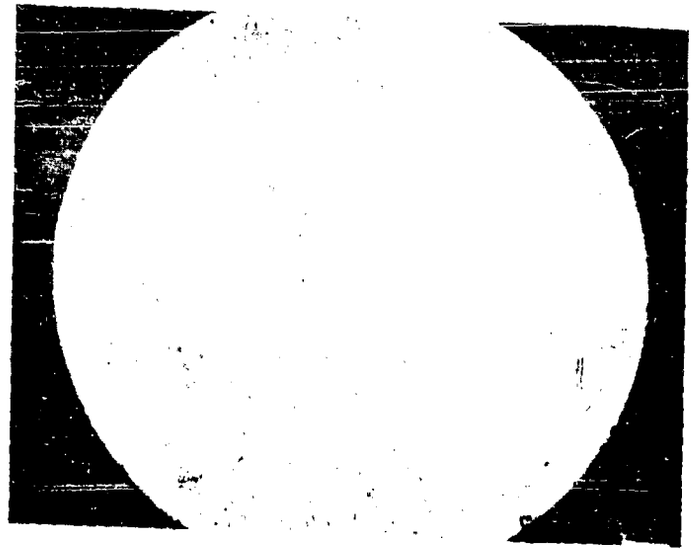


FIGURE 22. (U) CONJUNCTIVA CELLS BEFORE VIRUS INOCULATION.

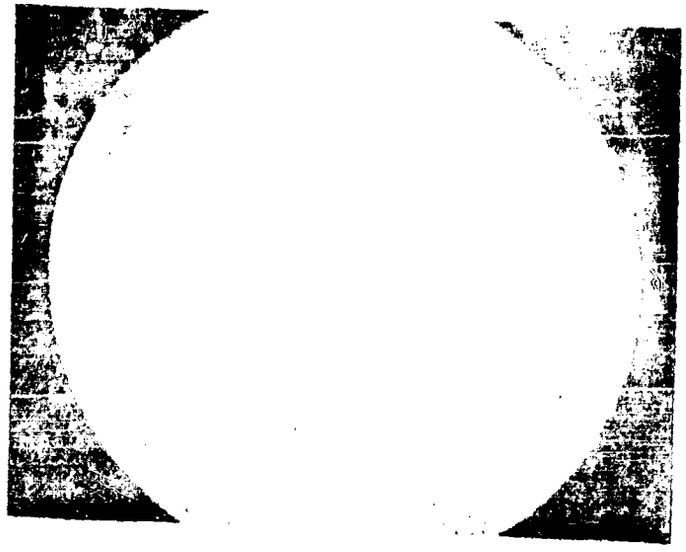


FIGURE 23. (U) CONJUNCTIVA CELLS THREE DAYS AFTER VIRUS INOCULATION.

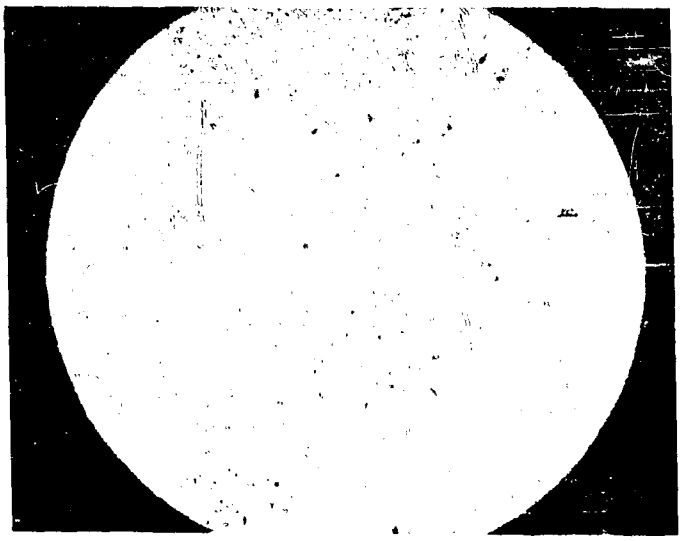


FIGURE 24. (U) MABEN CELLS BEFORE VIRUS INOCULATION.

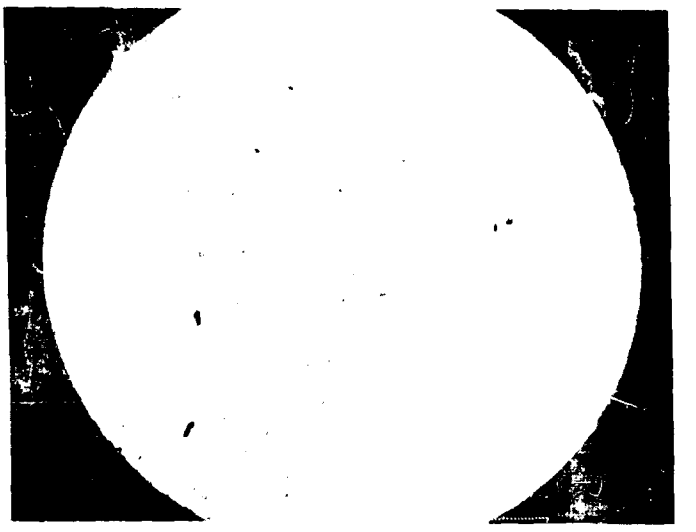
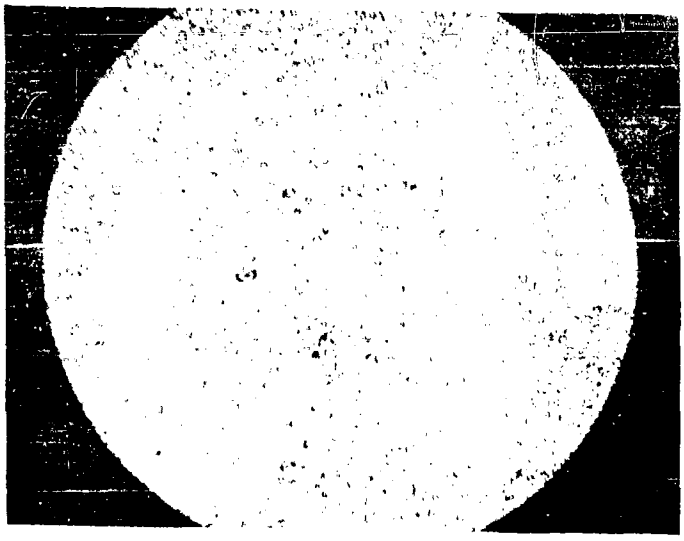


FIGURE 25. (U) MABEN CELLS THREE DAYS AFTER VIRUS INOCULATION.



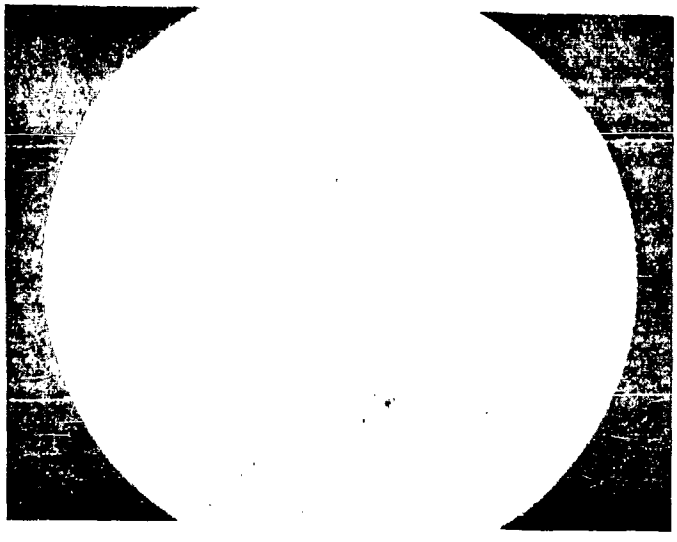


FIGURE 27. (U) KB CELLS THREE DAYS AFTER VIRUS INOCULATION.

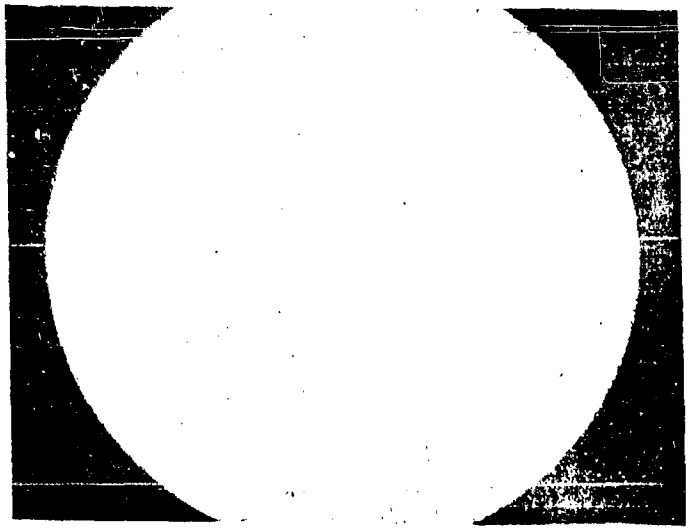


FIGURE 28. (U) INTESTINE CELLS BEFORE VIRUS INOCULATION.

FIGURE 29. (U) INTESTINE CELLS THREE DAYS AFTER VIRUS INOCULATION.

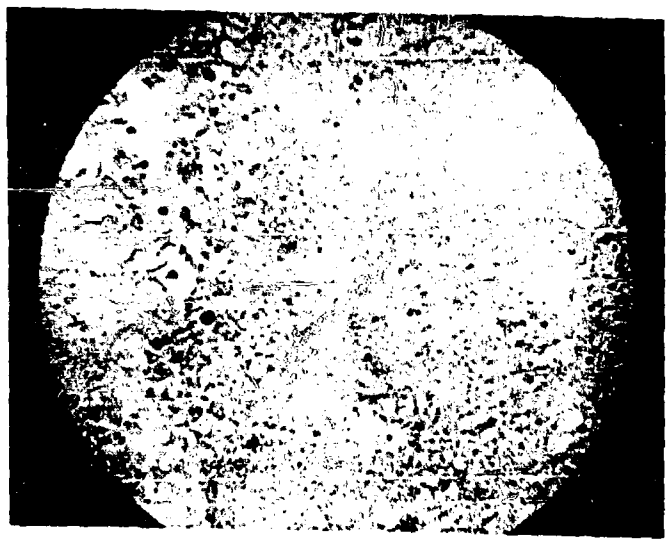


FIGURE 30. (U) HELA CELLS BEFORE VIRUS INOCULATION.

FIGURE 31. (U) HELA CELLS THREE DAYS AFTER VIRUS INOCULATION.

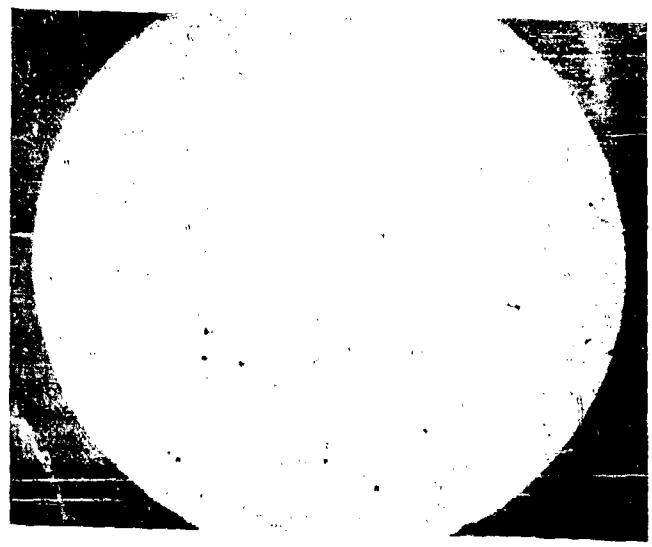


FIGURE 32. (U) BOVINE MUSCLE CELLS BEFORE VIRUS INOCULATION.

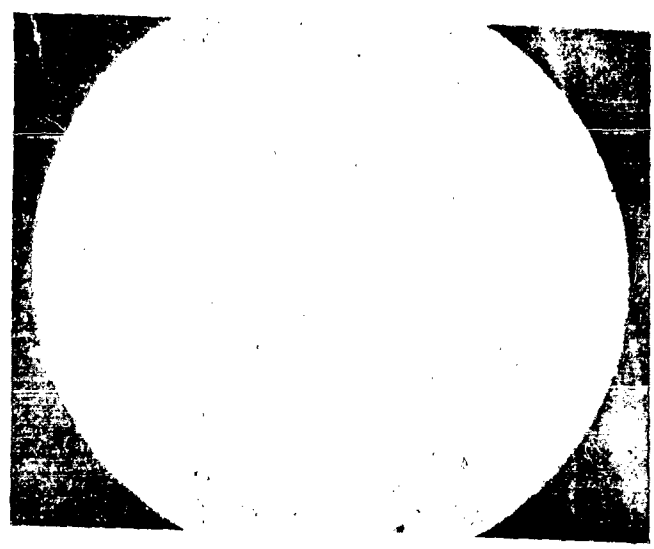


FIGURE 33. (U) BOVINE MUSCLE CELLS TWO DAYS AFTER VIRUS INOCULATION.

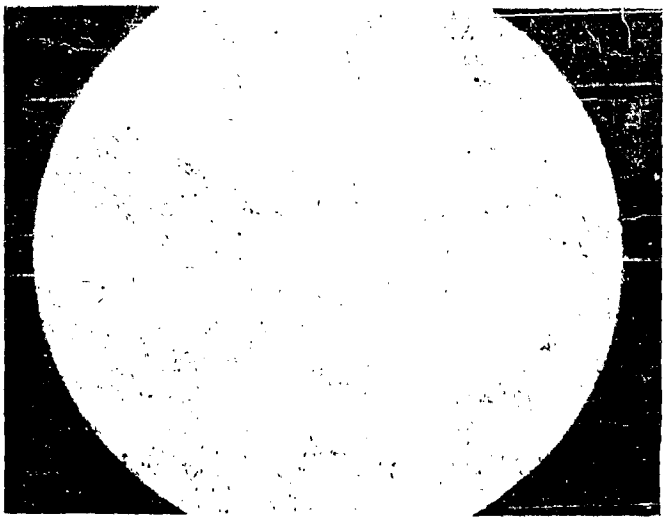


FIGURE 34. (U) MONKEY KIDNEY EPITHELIUM BEFORE VIRUS INOCULATION.

FIGURE 35. (U) MONKEY KIDNEY EPITHELIUM TWO DAYS AFTER VIRUS INOCULATION.

TABLE XXX. (U) PROPAGATION OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF HeLa CELLS

INOCULUM	TISSUE CULTURE PASSAGE	FLUID CHANGE	AGE IN CULTURE, days	CALCULATED DILUTION FROM SOURCE	INFECTIVITY AS INDICATED BY CAM INOCULATION, IU per ml
20 Per Cent CAM Virus Susp.	1	1	2	$10^{-5.3}$	7.8×10^{2}
		2	5	$10^{-6.6}$	3.3×10^{3}
		3	7	$10^{-7.9}$	6.6×10^{4}
		4	9	$10^{-9.2}$	1.4×10^{5}
1st T.C.[a] Generation 3rd Fluid Change	2	1	12	$10^{-9.2}$	1.2×10^{4}
		2	14	$10^{-10.5}$	2.0×10^{4}
		3	16	$10^{-11.8}$	5.1×10^{6}
2nd T.C Generation 3rd Fluid Change	3	1	19	$10^{-13.1}$	1.5×10^{4}
		2	20	$10^{-14.4}$	2.0×10^{5}
3rd T.C. Generation 2nd Fluid Change	4	1	22	$10^{-15.7}$	2.2×10^{3}
		2	23	$10^{-17.0}$	2.0×10^{4}

a. Tissue culture.

TABLE XXXI. (U) PROPAGATION OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF KB CELLS

INOCULUM	TISSUE CULTURE PASSAGE	FLUID CHANGE	AGE IN CULTURE, days	CALCULATED DILUTION FROM SOURCE	INFECTIVITY AS INDICATED BY CAM INOCULATION, IU per ml
20 Per Cent CAM Virus Susp.	1	1	2	$10^{-5.3}$	4.2×10^{2}
		2	5	$10^{-6.6}$	5.8×10^{2}
		3	7	$10^{-7.9}$	1.0×10^{5}
		4	9	$10^{-9.2}$	1.2×10^{6}
1st T.C.[a] Generation 3rd Fluid Change	2	1	12	$10^{-9.2}$	1.9×10^{4}
		2	14	$10^{-10.5}$	4.1×10^{4}
		3	16	$10^{-11.8}$	9.0×10^{5}
2nd T.C. Generation 3rd Fluid Change	3	1	19	$10^{-13.1}$	1.8×10^{4}
		2	20	$10^{-14.4}$	4.6×10^{5}
3rd T.C. Generation 2nd Fluid Change	4	1	22	$10^{-15.9}$	1.6×10^{4}
		2	23	$10^{-17.0}$	3.0×10^{4}
4th T.C. Generation 2nd Fluid Change	5	1	28	$10^{-18.3}$	6.6×10^{4}

a. Tissue culture.

TABLE XXX. (U) PROPAGATION OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF HeLa CELLS

INOCULUM	TISSUE CULTURE PASSAGE	FLUID CHANGE	AGE IN CULTURE, days	CALCULATED DILUTION FROM SOURCE	INFECTIVITY AS INDICATED BY CAM INOCULATION, IU per ml
20 Per Cent CAM Virus Susp.	1	1	2	$10^{-5.3}$	7.8×10^{2}
		2	5	$10^{-6.6}$	3.3×10^{3}
		3	7	$10^{-7.9}$	6.6×10^{4}
		4	9	$10^{-9.2}$	1.4×10^{5}
1st T.C.[a] Generation 3rd Fluid Change	2	1	12	$10^{-9.2}$	1.2×10^{4}
		2	14	$10^{-10.5}$	2.0×10^{4}
		3	16	$10^{-11.8}$	5.1×10^{6}
2nd T.C Generation 3rd Fluid Change	3	1	19	$10^{-13.1}$	1.5×10^{4}
		2	20	$10^{-14.4}$	2.0×10^{5}
3rd T.C. Generation 2nd Fluid Change	4	1	22	$10^{-15.7}$	2.2×10^{3}
		2	23	$10^{-17.0}$	2.0×10^{4}

a. Tissue culture.

TABLE XXXII. (U) PROPAGATION OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF L CELLS

INOCULUM	TISSUE CULTURE PASSAGE	FLUID CHANGE	AGE IN CULTURE, days	CALCULATED DILUTION FROM SOURCE	INFECTIVITY AS INDICATED BY CAM INOCULATION, IU per ml
20 Per Cent CAM Virus Susp.	1	1	2	$10^{-5.3}$	5.4×10^{2}
		2	5	$10^{-6.6}$	3.9×10^{3}
		3	7	$10^{-7.9}$	2.6×10^{4}
		4	9	$10^{-9.2}$	3.2×10^{4}
1st T.C.[a] Generation 3rd Fluid Change	2	1	12	$10^{-9.2}$	2.6×10^{4}
		2	14	$10^{-10.5}$	6.0×10^{3}
		3	16	$10^{-11.8}$	1.3×10^{5}
2nd T.C. Generation 3rd Fluid Change	3	1	19	$10^{-13.1}$	4.4×10^{3}
		2	20	$10^{-14.4}$	2.2×10^{4}
3rd T.C. Generation 2nd Fluid Change	4	1	22	$10^{-15.7}$	8.8×10^{3}
		2	23	$10^{-17.0}$	8.0×10^{4}

a. Tissue culture.

TABLE XXXIII. (U) SUMMARY OF PROPAGATION OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF HUMAN AND ANIMAL TISSUES

CELL LINE	NO. OF TISSUE CULTURE PASSAGES	CUMULATIVE LOG OF DILUTION OF ORIGINAL VIRAL INOCULUM	INFECTIVITY AS INDICATED BY CAM INOCULATIONS, IU per ml
HeLa	4	17.0	2.0×10^4 [a]
KB	4	18.3	6.6×10^4
Maben	5	15.7	9.0×10^4
Liver	4	17.0	4.8×10^2
Bovine Kidney	4	15.7	2.4×10^1
Conjunctiva	4	18.3	2.1×10^4
Monkey Kidney	4	15.7	2.0×10^3
L	4	17.0	8.0×10^4
Dog Kidney	4	15.7	2.0×10^2
Control (Virus in cell-free medium)	1	7.9	4.0×10^0

a. The initial inoculum at a final dilution of $10^{-5.3}$ per tube contained 1.1×10^3 infectious units per ml.

b. (C) Growth Curves

(U) In addition to the method just described for obtaining evidence of viral propagation in tissue culture, a growth curve study was made with several untested cell strains, human intestine, bovine muscle, S-180, J-111, D-189 and Detroit-116P. In Figure 36, viral growth curves are depicted with human intestine, bovine muscle and S-180 cell strains. Virus titers showed an initial decline for two or more days in these cultures before increased infectivity was evident. The maximum virus titers obtained from these cultures were: a 600-fold increase from intestine cells in nine days, 45-fold increase from bovine muscle cells in eight days, and approximately tenfold increase from S-180 cells in five days. A shortage of infected cultures terminated the experiment with intestine cells at the peak growth period and limited the number of assays for the S-180 virus growth curve. The virus control in cell-free medium declined in titer so that virus could not be detected at five days.

(C) Viral growth in the J-111 cell strain after inoculation with different dilutions of virus is plotted in Figure 37. It is evident that the virus propagated in this cell strain when appropriate inocula were employed. With viral dilutions of 10^{-1} and 10^{-3} a typical growth curve was noted which reached a peak level on the sixth day. A titer of 1.0×10^{8} infectious units per milliliter was obtained with the 10^{-1} dilution of inoculum which was equivalent to viral yields obtained from CAM inoculation of eggs. Undiluted inoculum gave only a 0.2 log virus increase over that of the initial concentration at its peak period of growth. For some reason this high concentration of initial inoculum did not exhibit a typical growth pattern, perhaps because of rapid destruction of cells by the high virus inoculum. With the most dilute inoculum (10^{-5}) an initial increase of virus was followed by a second lag period. A second increase of the agent was then observed. Further observations were not possible because the experiment was terminated by a shortage of tubes.

(U) Figure 38 shows propagation of the virus in Detroit-116P and D-189 cell strains. As in findings with S-180 cells, peak viral concentrations were obtained on the fourth to fifth days. With both cell strains, virus titers were greater than 1.0×10^{8} infectious units per milliliter and showed an approximate increase of 1000-fold over that of the initial inoculum. Viral growth slowly declined after this peak period of multiplication.

(U) An additional growth curve study was made with the Maben cell strain in which virus had been shown previously to propagate by serial passage. The growth curves after inoculation of different dilutions of the agent are shown in Figure 39.

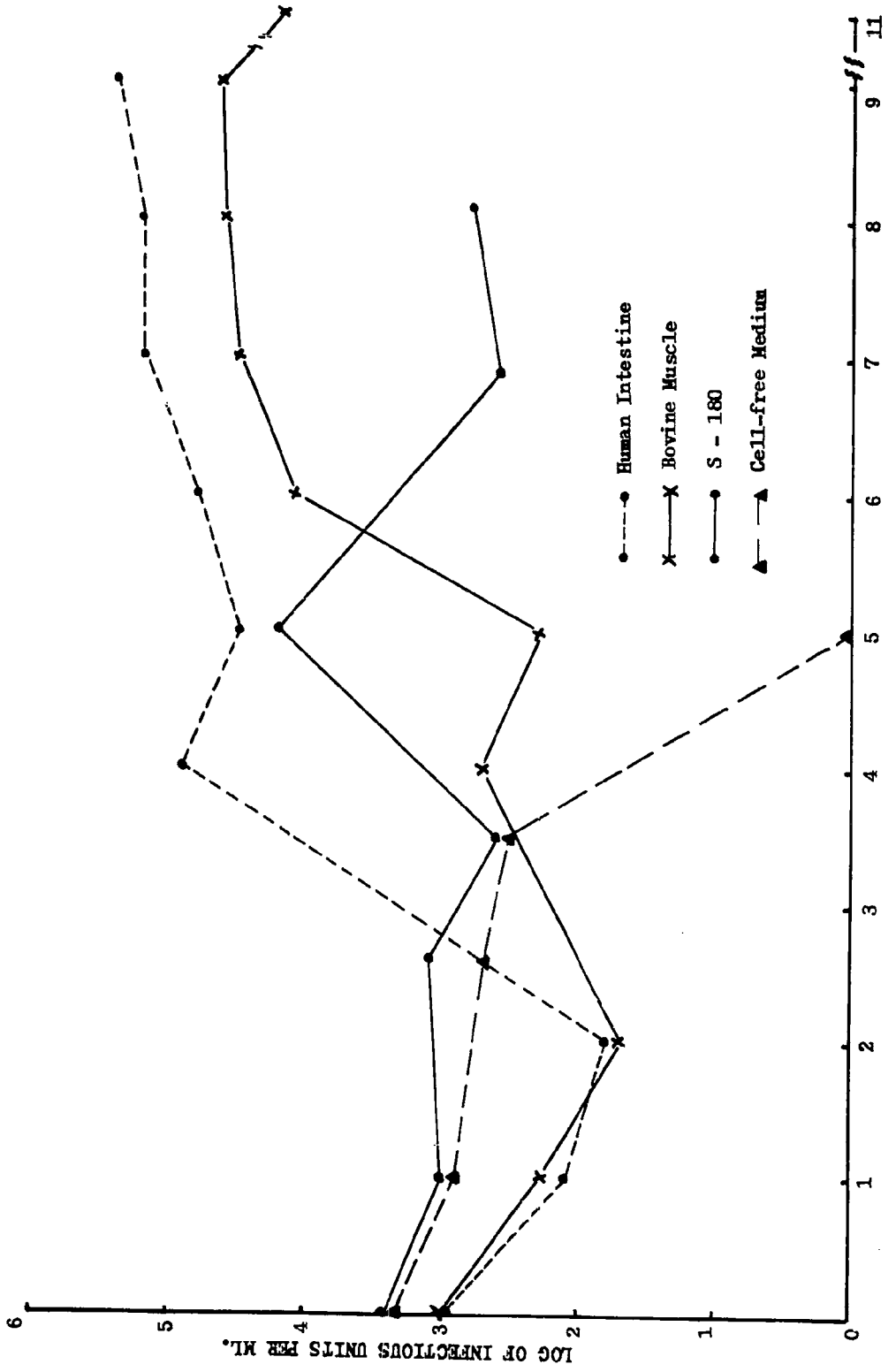


FIGURE 36. (U) GROWTH CURVES OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF HUMAN INTESTINE, BOVINE MUSCLE, AND S-180 CELL STRAINS.

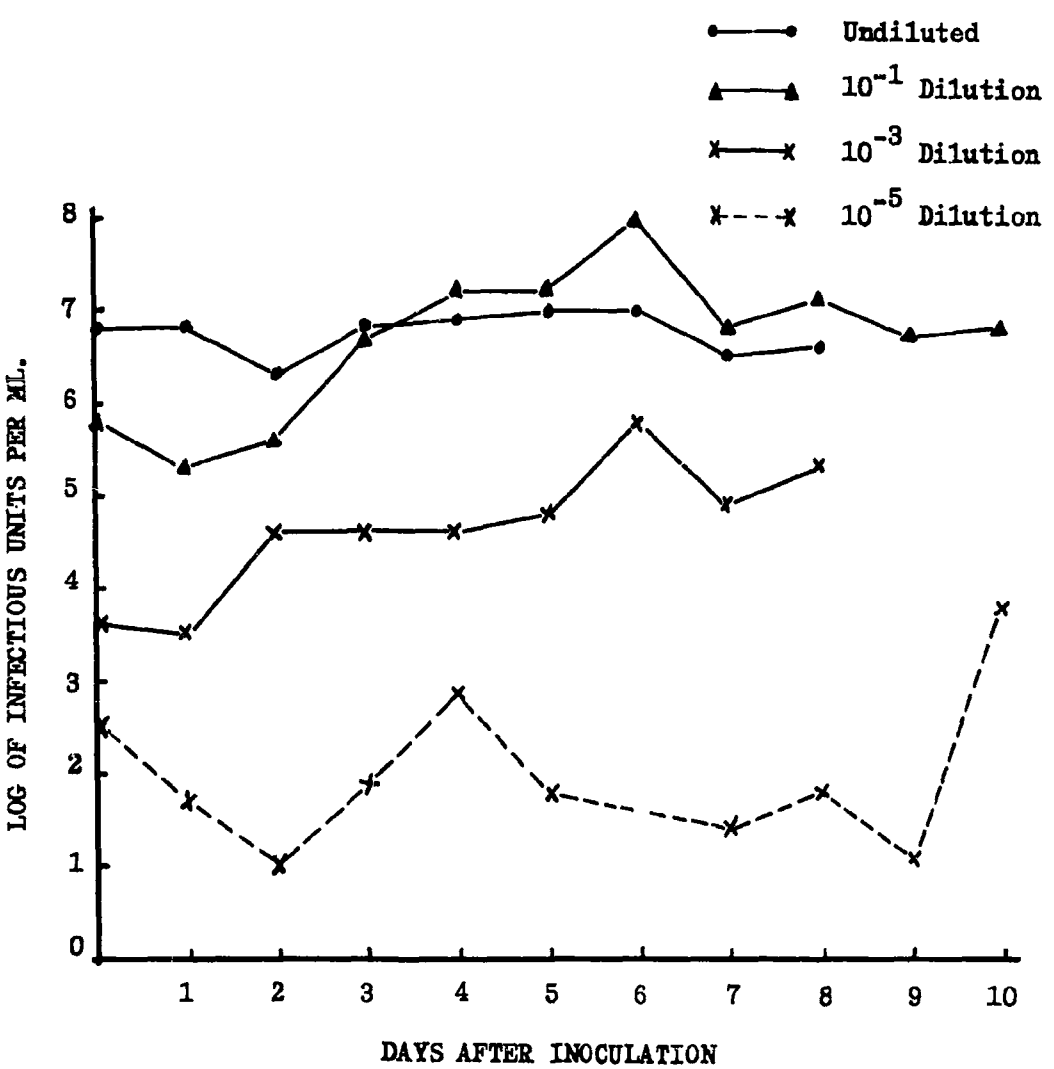


FIGURE 37. (U) GROWTH CURVES OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF J-111 CELL STRAIN WITH VARIED DILUTIONS OF INOCULUM.

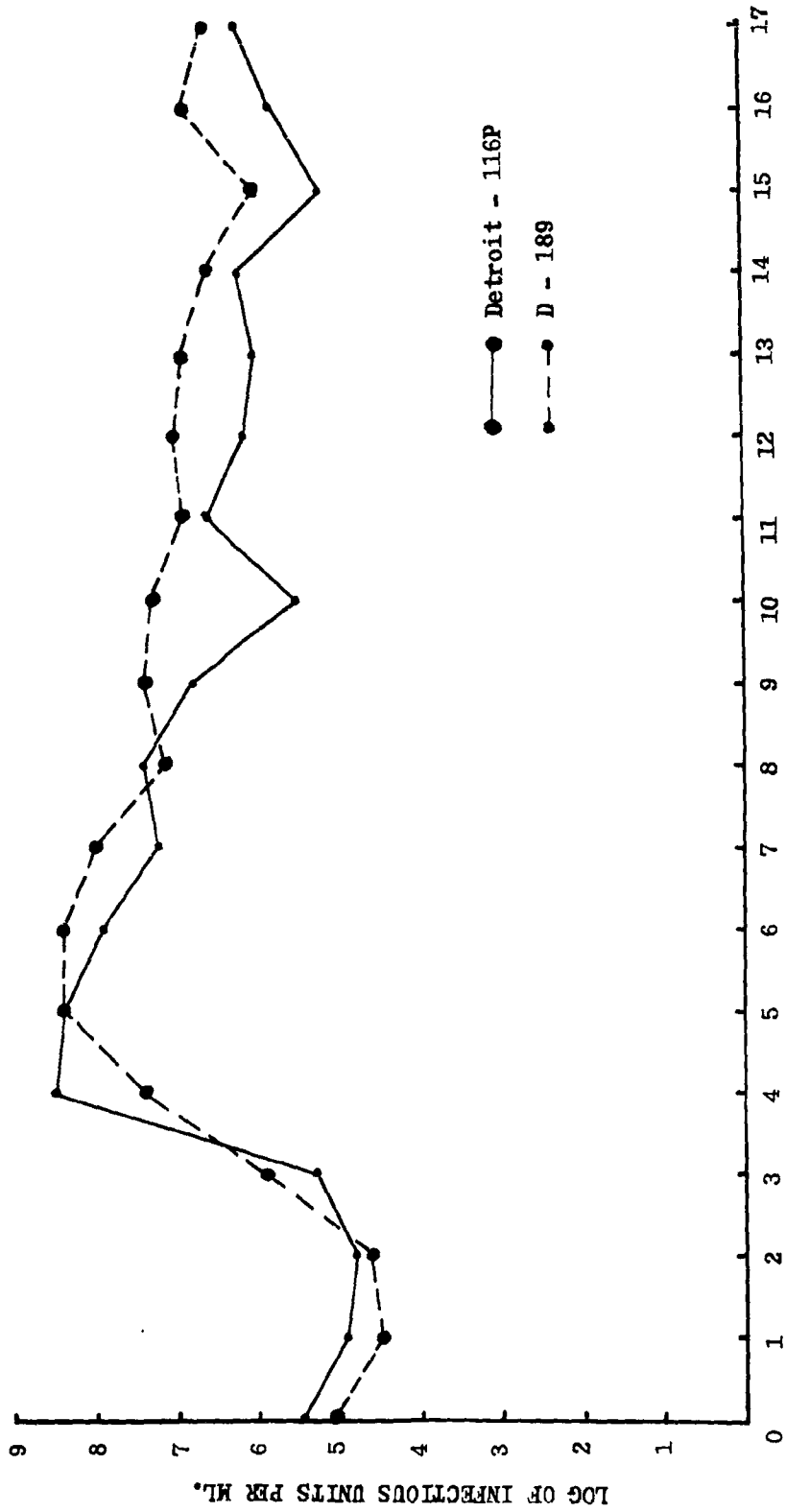


FIGURE 38. (U) GROWTH CURVES OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF D-189 AND DETROIT-116P CELL STRAINS.

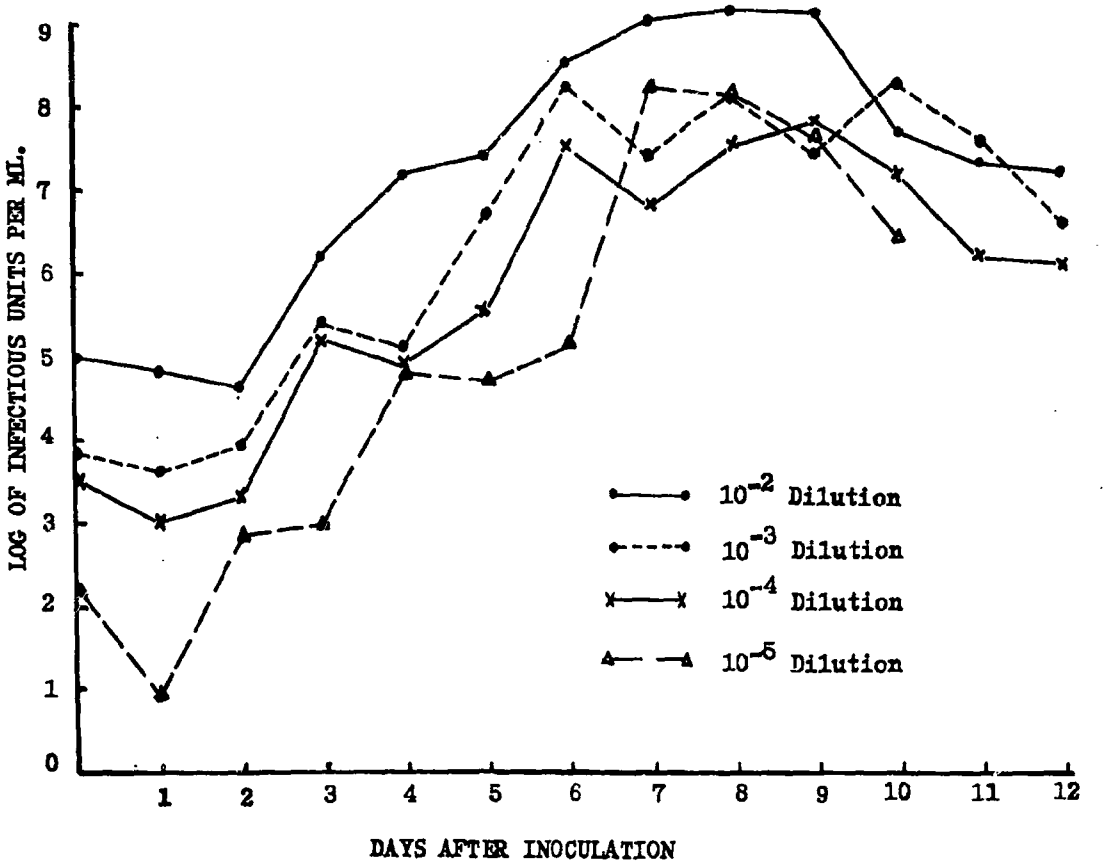


FIGURE 39. (U) GROWTH CURVES OF VARIOLA VIRUS IN ROLLER TUBE CULTURES OF MABEN CELL STRAIN WITH VARIED DILUTIONS OF INOCULUM.

(C) It is evident from the growth curves that the virus propagated in the Maben cell strain. With all dilutions used there was a lag period of one to two days, followed by several days of multiplication. Peak growth periods were noted from the sixth to ninth days. Three of the dilution inocula (10^{-2}, 10^{-3}, and 10^{-5}) showed viral yields greater than 1.0×10^{8} infectious units per milliliter. Most surprising was the finding that a 10^{-2} dilution of inoculum showed a virus yield of 1.2×10^{9} infectious units per milliliter. This titer was noted for three consecutive days. It was higher than any other titer obtained in this laboratory from CAM inoculation of eggs with the agent. The data from these preliminary growth curve studies indicated that a potential method was available for propagating variola virus in tissue culture.

3. (U) Quantitative Relationship of Virus and Antibody in Tissue Culture

(U) With certain viruses a linear log-log relationship exists between different amounts of virus and immune serum.[37 - 40] The existence of a similar relationship with variola virus and serum in tissue culture might offer a quantitative method for determining the antibody content of serum.

(U) "Box" titrations were carried out in which various quantities of virus were added to different amounts of immune monkey serum (heat-inactivated at 56°C for 30 minutes). Immune serum was obtained from a convalescent M. rhesus monkey which had been inoculated intranasally with variola virus 20 days previously. The mixtures of virus and serum were incubated at 25°C for one hour. Each of five culture tubes was inoculated with 0.1 ml of one of the mixtures. Viruses were titrated in tissue culture at the same time to determine the quantity of virus neutralized by the various amounts of serum. Tubes were incubated at 35°C in a roller drum and the serum dilution endpoints determined at four days. The endpoint was the greatest quantity of virus completely neutralized by a serum dilution, i.e., no cellular degeneration by virus was evident.

(U) A similar relationship was obtained with variola virus in tissue culture as shown by the straight-line logarithmic correlation of slope 1.0 between the serum titer and the quantity of virus neutralized (Figure 40). The relationship was identical in three randomly selected cell cultures. To attain a tenfold increase in neutralized virus, the required serum quantity was increased tenfold. Correspondingly, a tenfold decrease in serum caused a similar decrease of neutralized virus.

(U) The usual practice of measuring serum for variola-neutralizing antibody involves the inoculation of mixtures of virus and serum on the CAMs of embryonated eggs and determining the highest dilution of serum that permits an average pock reduction of 50 per cent or greater. The CAM technique requires experience to avoid inherent errors and at best gives somewhat variable results. An additional source of error is introduced into the method by the lack of proportionality between virus dilutions and pock counts in the presence of serum.[41] As pointed out by Shaffer and Enders,[42] a tenfold change in antibody concentration may not alter the pock count. The relationship demonstrated between mixtures of variola virus and serum offers the possibility of using a tissue culture neutralization test to determine serum antibody titers.

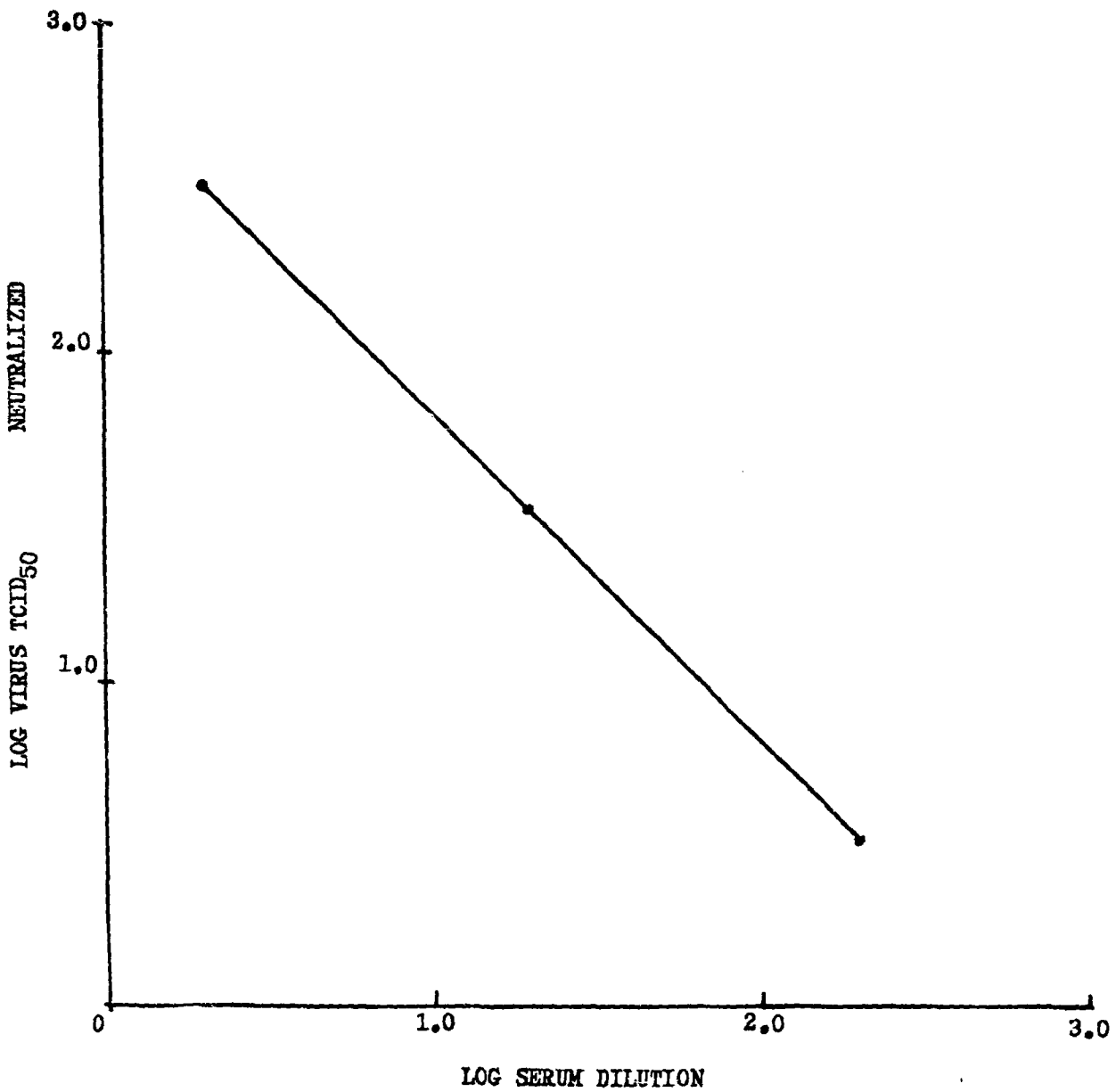


FIGURE 40. (U) QUANTITATIVE RELATIONSHIPS BETWEEN VARIOLA VIRUS AND SPECIFIC-IMMUNE MONKEY SERUM IN HELA, KB, AND MABEN TISSIE CULTURE NEUTRALIZATION TEST. THE PLOTTED POINTS ARE IDENTICAL FOR EACH CELL STRAIN TESTED.

IV. (S) DISCUSSION

(C) The primary need of this project at its initiation was a method for quantitating virus infectivity. Although a basic method was available, that of inoculation of chorioallantoic membranes and enumeration of lesions or pocks induced by the virus, the incidence of nonspecific lesions created difficulty. In addition, the method had to be adapted to work in a Class III cabinet. A rapid technique was successfully developed to prepare eggs for chorioallantoic membrane inoculation which practically eliminated the presence of nonspecific lesions and permitted accurate determination of the virus content of experimental preparations.

(C) The embryonated egg not only served as a means of quantitating agent viability but also was used as a virus source for experimental purposes. Large-scale production of variola virus from the chorioallantoic membrane of eggs may not be feasible and other sources may be required.

(S) The preliminary information on viral multiplication resulting in high titer yields with tissue cultures of human cell lines seems promising for exploring the feasibility of large-scale agent production. Virus titers obtained from tissue cultures were equivalent to or greater than those achieved in eggs. The findings on viral cytopathogenicity offer additional methods of assaying the agent and of immunological testing. More important is the possibility, yet to be investigated, of enhancing virus virulence by repeated passage in human cultivated cells of the lung or skin.

(C) The virus has shown good stability to drying and storage at lower temperatures (-25° to -16°C), both in the liquid and dried forms. The stability in the dried form was better than in the liquid at room temperature (26°C) and at 4°C. The use of suitable diluents may further prolong the viability of both liquid and dried agent at higher storage temperatures.

(S) Limited progress has been made in defining the optimum conditions for aerosol dissemination of viruses. Some preliminary data were obtained to show that the amount of virus recovered could be significantly increased by suitable impinger fluids. However, extensive testing is necessary to define the conditions of temperature and humidity needed to maintain optimal viability of both liquid and dried agent in an aerosol.

(S) Aerosol infectivity studies have shown the liquid aerosolized agent to be infectious for monkeys. The respiratory ID_{50} has not yet been determined because of the need for more precise characterization of variola virus aerosols. The particle size was estimated to be about five microns by direct microscopic examination of membrane filters. This size of particle was apparently suitable for lodging in the deep lung tissue of monkeys, as evidenced by the findings from study on the pathogenesis of the disease in monkeys.* Thus far, no animals have been exposed to aerosols of dried preparations.

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* Report in preparation, "Pathogenesis of Variola in Macaca irus Monkeys."

(C) Limited animal infectivity studies showed that the disease pattern in monkeys was similar to that of human infections with the exception of a shorter incubation period in monkeys. At present, monkeys are the only available host as indicators of infection. There was evidence to suggest that M. irus were more susceptible than M. rhesus monkeys but further tests are needed to confirm this preliminary observation. Other species of monkeys have been known to contract the disease,[34] and should be tested for susceptibility to this agent.

(S) Although six of the seven variola virus strains were infective for monkeys, additional experimentation is required to evaluate and select the best of these strains from the standpoint of production, stability, and animal infectivity. Most of the experimental information in this report was obtained with the Yamada strain. It is conceivable that the few egg passages made with this virus strain may have altered its capability of producing disease for both monkeys and humans. However, it is equally possible that variola virus possesses marked antigenic stability and unaltered virulence for man and monkeys even when passaged in an abnormal host. Information on this phase of the virus is of paramount importance for its successful development as an agent.

(S) A study is planned to determine the effectiveness of standard immunization procedures in preventing the airborne disease. The relationship between the time interval after vaccination and the amount of inhaled variola virus necessary to overcome immunity should be valuable to the BW program.

V. (S) CONCLUSIONS

(S) Variola virus has in general fulfilled the requirements of the screening phase which are necessary in a candidate agent for further BW development. It has been produced in reasonably high titers from eggs and in tissue culture, dried without viability loss, found to possess good storage stability in both liquid and dried forms, and to be infective for monkeys by inhalation of aerosolized virus.

(U) LITERATURE CITED

1. Morbidity and Mortality Weekly Report, PHS, U. S. Department of Health, Education, and Welfare, Vol. 6, No. 31, August 9, 1957.

2. Downie, A. W., and Dumbell, K. R.: "The Isolation and Cultivation of Variola Virus on the Chorio-Allantoic of Chick Embryos," J Path Bact, 59:1-2:189-198, January - April 1947.

3. Goodpasture, E. W.; Woodruff, A. M.; and Buddingh, G. J.: "The Cultivation of Vaccine and Other Viruses in the Chorioallantoic Membrane of Chick Embryos," Science, 74:1919:371-372, October 1931.

4. Bengtson, I. A., and Dyer, R. E.: "Cultivation of the Virus of Rocky Mountain Spotted Fever in the Developing Chick Embryo," Public Health Report, Washington, 50:43:1489-1498, October 1935.

5. Alexander, R. A.: "Studies on the Neurotropic Virus of Horse-Sickness: VI. Propagation in the Developing Chick Embryo," Onderstepoort J Vet Sci Animal Ind, 11:1:9-19, July 1938.

6. Stevenson, W. D. H., and Butler, G. G.: "Studies on the Cultivation of Vaccinia on the Chorio-Allantoic Membranes of Chick Embryos," Ministry of Health Reports on Public Health and Medical Subjects, His Majesty's Stationery Office, London, No. 87, 1939.

7. Burnet, F. M., and Faris, D. D.: "The Technique of Quantitative Chorioallantoic Virus Titration," J Bact, 44:2:241-248, August 1942.

8. Dunham, W. B.: "An Egg Inoculator and Shell Membrane Teaser for Virus Culture," Science, 95:2476:609, June 1942.

9. Beveridge, W. I. B., and Burnet, F. M.: "The Cultivation of Viruses and Rickettsiae in the Chick Embryo," Med Research Council (Brit), Special Report Ser No. 256, 1946.

10. Scott, T. F. McN.: Diagnostic Procedures for Virus and Rickettsial Diseases, 1st Edition, American Public Health Association, Publication Office, New York, 1948. pp. 243-266.

11. Keogh, E. V.: "Titration of Vaccinia Virus on the Chorio-Allantoic Membrane of the Chick Embryo and Its Application to Immunological Studies of Neuro-Vaccinia," J Path Bact, 43:3:441-454, November 1936.

12. Reid, D. B. W.; Crawley, J. F.; and Rhodes, A. J.: "A Study of Fowl Pox Virus Titration on the Chorio-Allantoic by the Pock Counting Technique," J Immunol, 63:2:165-171, October 1949.

13. Overman, J. R., and Tamm, I.: "Quantitative Titration of Vaccinia Virus on the Chorioallantoic Membrane," J Immunol, 76:3:228-236, March 1956.

14. Clark, E., and Nagler, F. P. O.: "The Range of Susceptible Cells with Special Reference to Agglutination by Vaccinia Virus," Australian J Exptl Biol Med Sci, 21:2:103-106, June 1943.

15. Andrewes, C. H., and Elford, W. J.: "Observations on Anti-Phage Sera: I. The 'Percentage Law'," Brit J Exptl Path, 14:6:367-376, December 1933.

16. Morgan, J. F.; Morton, H. J.; and Parker, R. C.: "Nutrition of Animal Cells in Tissue Culture: I. Initial Studies on A Synthetic Medium," Proc Soc Exptl Biol Med, 73:1-8, January - April 1950.

17. Younger, J. S.: "Monolayer Tissue Cultures: I. Preparation and Standardization of Suspensions of Trypsin - Dispersed Monkey Kidney Cells," Proc Soc Exptl Biol Med, 85:2:202-205, February 1954.

18. Reed, L. J., and Muench, M.: "Simple Method of Estimating Fifty Per Cent Endpoints," Am J Hyg, 27:493-497, May 1938.

19. Gey, G. O.; Coffman, W. D.; and Kubicek, M. T.: "Tissue Culture Studies of the Proliferative Capacity of Cervical Carcinoma and Normal Epithelium," Cancer Research, 12:4:264-265, April 1952.

20. Eagle, H.: "Propagation in a Fluid Medium of a Human Epidermoid Carcinoma Strain KB," Proc Soc Exptl Biol Med, 89:362-364, May - September 1955.

21. Henle, G., and Deinhardt, F.: "The Establishment of Strains of Human Cells in Tissue Culture," J Immunol, 79:1:54-59, July 1957.

22. Berman, L., and Stulberg, C. S.: "Eight Culture Strains (Detroit) of Human Epithelial-Like Cells," Proc Soc Exptl Biol Med, 92:4:730-735, August - September 1956.

23. Osgood, E. E., and Brooke, J. H.: "Continuous Tissue Culture of Leukocytes from Human Leukemic Bloods by Application of 'Gradient' Principles," Blood, 10:1010-1022, October 1955.

24. Leighton, J.; Kline, I.; and Orr, H. C.: "Transformation of Normal Human Fibroblasts into Histologically Malignant Tissue In Vitro," Science, 123:319:502-503, March 1956.

25. Chang, R. S.: "Continuous Subcultivation of Epithelial-Like Cells from Normal Human Tissues," Proc Soc Exptl Biol Med, 87:440-443, October - December 1954.

26. Foley, G. E., and Droplet, B. P.: "Sustained Propagation of Sarcoma 180 in Tissue Culture," Proc Soc Exptl Biol Med, 92:347-352, May - September 1956.

27. Sanford, K. K.; Earle, W. R.; and Likely, G. D.: "The Growth In Vitro of Single Isolated Tissue Cells," J Natl Cancer Inst, 9:3:229-246, December 1948.

28. Frisch, A. W.; Jentoft, V.; Barger, R.; and Losli, E. J.: "A Human Epithelium-Like Cell (Maben) Derived from an Adenocarcinoma of Lung," Am J Clin Path, 25:10:1107-1112, October 1955.

29. Ginsberg, H. S., and Horsfall, F. L., Jr.: "Quantitative Aspects of the Multiplication of Influenze A Virus in the Mouse Lung," J Exptl Med, 95:2:135-145, February 1952.

30. Scott, T. F. McN.; Coriell, H. B.; and Gray, A.: "The Growth Curve of the Virus of Herpes Simplex on the Chorioallantoic Membrane of the Embryonated Hen's Egg," J Immunol, 71:3:134-144, September 1953.

31. Kilbourne, E. D.; and Horsfall, F. L., Jr.: "Increased Virus in Eggs Injected with Cortisone," Proc Soc Exptl Biol Med, 76:116-118, January - April, 1951.

32. Kilbourne, E. D.: "Reactivation of Non-Infective Virus in a Cortisone-Injected Host," J Exptl Med, 101:4:437-450, April 1955.

33. Duran-Reynals, F.: "Tissue Permeability and the Spreading Factors in Infection," Bact Revs, 6:4:197-252, December 1942.

34. Andrewes, C. H.; Arkwright, J. A.; Bedson, S. P.; Blaxall, F. R.; et al: A System of Bacteriology in Relation to Medicine, His Majesty's Stationery Office, London, 7:1930. pp. 84-132.

35. Brinckerhoff, W. R., and Tyzzer, E. E.: "Studies Upon Experimental Variola and Vaccinia in Quadrumana," J Med Research, 14:2:213-359, January 1906.

36. Mayyasi, S. A.; Schuurmans, D. M.; and Brown, A.: "A Rapid Method for the Titration of Vaccinia Virus and Its 'Neutralizing' Antibody in Tissue Culture," Bact Proc, 66, 1957.

37. Horsfall, F. L., Jr.: "Neutralization of Epidemic Influenza Virus: The Linear Relationship Between the Quantity of Serum and the Quantity of Virus Neutralized," J Exptl Med, 70:2:209-222, August 1939.

38. Morgan, I. M.: "Quantitative Study of the Neutralization of Western Equine Encephalomyelitis Virus by Its Antiserum and the Effect of Complement," J Immunol, 50:6:359-371, June 1945.

39. Bell, J. E.: "Experimental Studies of Variables in Neutralization Tests with Lansing Poliomyelitis Virus," Am J Hyg, 48:3:381-393, November 1948.

40. Ledinko, N.; Riordan, J. T.; and Melnick, J. L.: "Multiplication of Poliomyelitis Viruses in Tissue Cultures of Monkey Testes: I. Growth Curves of Type I (Brunhilde) and Type 2 (Lansing) Strains and Description of a Quantitative Neutralization Test," Am J Hyg, 55:3:323-338, May 1952.

41. Jawetz, E., and Coleman, V. R.: "Studies on Herpes Simple Virus: III. The Neutralization of Egg-Adapted Herpes Virus by Human Sera In Ovo," J Immunol, 68:6:645-653. June 1952.

42. Shaffer, M. F., and Enders, J. F.: "Quantitative Studies on the Infectivity of the Virus of Herpes Simplex for the Chorioallantoic Membrane of the Chick Embryo, Together with Observations on the Inactivation of the Virus by Its Specific Antiserum," J Immunol, 37:4:383-411, October 1939.

(U) REFERENCES

Blank, H. and Rake, G.: Viral and Rickettsial Diseases of the Skin, Eye, and Mucous Membranes of Man, Little, Brown and Company, Boston, 1955.

Diagnostic Procedures for Virus and Rickettsial Diseases, Second Edition, American Public Health Association, New York City, 1956.

Rooyen, C. E. and Rhodes, A. J.: Virus Diseases of Man, Thomas Nelson and Sons, New York, 1948.

Smadel, J. E.: Viral and Rickettsial Infections of Man, Second Edition, T. B. Lippincott Company, Philadelphia, 1952.

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